Effects of sublethal nitrite concentrations on the metabolism of the sea bass, *Lates calcarifer*.

by

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ABSTRACT

Changes in metabolism of an euryhaline teleost, Lates calcarifer, in response to ambient nitrite exposure were studied.

Acute toxicity tests revealed that the 96 hour median lethal concentrations of nitrite for Lates calcarifer in fresh water, 15 $^{o}/_{oo}$ sea water and 32 $^{o}/_{oo}$ sea water were 14.5 mgl^{-1} NO_2-N, 104 mgl^{-1} NO_2-N and 93 mgl^{-1} NO_2-N respectively. Nitrite, at relatively low concentrations, was shown to severely repress the growth of L. calcarifer in 32 $^{o}/_{oo}$ sea water.

Exposure to nitrite-containing sea water resulted in an elevation of blood nitrite which was accompanied by pronounced methemoglobinemia. A decrease in total hemoglobin content was also observed.

The blood of Lates calcarifer has a hyperbolic oxygen dissociation curve and a moderately low oxygen affinity. The oxygen dissociation curve of nitrite-treated fish shifts to the left, indicating an increased blood oxygen affinity. Moreover, nitrite-treated fish demonstrated a lowered arterial and venous blood oxygen tension, arterial and venous oxygen content, and blood oxygen capacity. On the other hand, the erythrocytes of L. calcarifer was found to possess the enzyme NADH-methemoglobin reductase which can convert methemoglobin back to hemoglobin. However, acute or prolonged nitrite exposure did not induce any change in its activity.

Exposure to a nitrite concentration of 10 mgl^{-1} NO_2-N for 3

days induced an elevation in serum sodium, serum potassium, and serum ammonia concentrations, and a decrease in serum lipid content. However, the tissue composition of liver, muscle, and brain was not affected. Hypoxic symptoms including lactate accumulation and glycogen depletion were observed upon exposure to nitrite at concentrations of or higher than 30 mgl^{-1} NO_2-N.

Acute exposure to 50 mgl^{-1} NO_2-N resulted in a marked decrease in liver ATP, ADP and AMP contents. Activities of branchial Na^+-K^+-ATPase, hepatic glycogen phosphorylase, glutamate-oxaloacetate transaminase and glutamate dehydrogenase were also decreased. In nitrite-treated fish, there is evidence for enhanced ammonia production probably via the purine nucleotide cycle. Considerable amount of urea was also generated probably through uricolysis.

The results obtained in the present study hopefully can contribute to mariculture and the understanding of the mechanisms of nitrite toxicity in fish.

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Table 2.1. Names of fish species mentioned in the text and tables

Scientific Name	Common Name
Anguilla anguilla	European eel
Brachydanio rerio	Zebrafish
Brycon cf. melanopterum	-
Carpiodes cyprinus	Quillback
Catostomus commersoni	White sucker
Channa maculata (Ophiocephalus maculatus)	Snakehead
Chanos chanos	Milkfish
Clarias lazera	-
Clarias mossambicus	-
Cottus bairdi	Mottled sculpin
Culaea inconstans	Brook stickleback
Cyprinus caprio	Common carp
Dicentrarchus labrax	-
Epinephalus akaara	Red grouper
Eptatretus stoutii	Hagfish
Gadus morhua	Atlantic cod
Gambusia affinis	Mosquitofish
Ictalurus melas	Black bullhead
Ictalurus punctatus	Channel catfish
Lagodon rhomboides	Pinfish
Lates albertianus	-
Lates calcarifer	Sea bass
Lepomis macrochirus	Bluegill
Micropterus salmoides	Largemouth bass
Mylio macrocephalus	Black sea bream
Oncorhynchus gorbuscha	Pink salmo
Oncorhynchus kisutch	Coho salmo
Oncorhynchus tschawytscha	Chinook salmon
Opsanus tau	-

Table 2.1. -- continued

Scientific Name	Common Name
Percina caprodes	Logperch
Phoxinus laevis	European minnow
Pimephales promelas	Fathead minnow
Poecilia reticulata	Guppy
Protopterus aethiopicus	African lungfish
Salmo clarki	Cutthroat trout
Salmo gairdneri	Rainbow (Steelhead) trout
Salmo salar	Atlantic salmo
Semaprochilodus insignis	-
Semotilus atromaculatus	Creek chub
Tautogolabrus adspersus	Cunner
Tilapia aurea	Blue tilapia
Tilapia esculenta	-
Tilapia zilli	-

Table 2.2. Different units for nitrite concentration and their equivalence

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Units:

1. parts per million (ppm)
2. milligram per litre (mgl^{-1})
3. millimolar (mM)

Their equivalence:

1 ppm NO_2	=	1 mgl^{-1} NO_2
1 mgl^{-1} NO_2	=	0.304 mgl^{-1} NO_2-N
1 mgl^{-1} NO_2-N	=	3.286 mgl^{-1} NO_2
1 mM NO_2	=	46 mgl^{-1} NO_2
1 mM NO_2-N	=	14 mgl^{-1} NO_2-N

CHAPTER ONE GENERAL INTRODUCTION

GENERAL INTRODUCTION

Nitrite is an intermediate product of nitrification in which ammonia is converted to nitrate. It is not considered as a severe environmental pollutant because of its trace amounts in most natural water systems and rapid oxidation to nontoxic nitrate under aerobic conditions. However, under some special circumstances such as water-reuse system and intensive fish culture, the nitrite concentration in the water may rise to a level which is toxic to aquatic animals.

Accumulation of nitrite is one of the most critical problems frequently encountered by fish culturists employing water-reuse systems to culture fish. Various types of biological filters have been used to reduce the concentration of ammonia, the primary nitrogenous excretory product of fish, as well as to control the environmental factors such as pH, temperature and dissolved oxygen. The nitrite level may increase significantly due to imbalances in the relative abundances of the nitrifying bacteria Nitrosomonas and Nitrobacter, posing serious hazard to fish growth and survival. The increasing need to develop intensive fish culture coupled with restricted availability of unpolluted water supplies have led to a growing interest in employing closed water-reuse systems for fish culture in many countries, the study of nitrite toxicity to fish thus becomes a relevant topic in the field of aquacultural sciences.

Nitrite toxicity has been studied in several fish species

(eg. Smith and Williams, 1974; Russo and Thurston, 1977; Palachek and Tomasso, 1984b; Wilmy et al., 1987) and tetrapod larvae (eg. Huey and Beitinger, 1980a, b). Lethal levels expressed as 96 hour median lethal concentrations (96 hr LC50s) for fishes range from 0.27 mgl^{-1} NO_2-N for rainbow trout (Russo et al., 1974) to 140 mgl^{-1} NO_2-N for largemouth bass (Palachek and Tomasso, 1984b) and these values are reported to be highly dependent upon water chemistry.

One major toxic effect of nitrite is the oxidation of hemoglobin to methemoglobin, a derivative incapable of binding oxygen (Bodansky, 1951). Unfortunately, the physiological and hematological factors associated with this response are poorly understood. Until present, no evidence has pointed methemoglobinemia as a direct cause of death in nitrite-exposed fish. Some proposed that mortality in nitrite-exposed channel catfish was due to tissue anoxia induced by methemoglobin (Huey et al., 1980) while others concluded that death in rainbow trout resulted from the toxic action of nitrite upon vital organs and not methemoglobinemia per se (Smith and Williams, 1974). Therefore, it is necessary to study more extensively the biochemical, physiological and hematological parameters of nitrite-exposed fish in order to deduce the real cause of death.

At present, the amount of published information on nitrite toxicity to fish is limited, and much of the available data is derived from tests on freshwater species such as rainbow trout and channel catfish. The information on nitrite toxicity to marine or euryhaline

fish remains sparse.

Research on the importance of the effect of water chemistry on nitrite toxicity has received increasing attention over the past few years. Increases in pH, chloride concentration, salinity and hardness have been shown to decrease nitrite toxicity in various freshwater fish species, however, data on the response of marine or euryhaline fish to such changes are unavailable.

Sea bass, Lates calcarifer, is an euryhaline teleost which is quite tolerant to a wide range of salinities and oxygen levels. It was chosen as the test species because this species is an economically important food fish that is commonly cultured in Hong Kong and many other South-east Asian countries. However, no nitrite toxicity information on this species is available. The present research was conducted to obtain acute and chronic nitrite toxicity data for the euryhaline teleost L. calcarifer and also outline its biochemical, hematological and physiological responses to nitrite exposure.

The data obtained in the study can give us a better understanding of the underlying mechanisms of nitrite toxicity on euryhaline fish and hopefully can contribute to the extension of rearing euryhaline or marine fish in a closed recirculating water system.

CHAPTER TWO. LITERATURE REVIEW

2.1. INTRODUCTION

Ammonia (NH_3), nitrite (NO_2^-) and nitrate (NO_3^-) are important water quality parameters in the sustenance of aquatic life. Ammonia is one of the most important pollutants in the aquatic environment because of its relatively highly toxic nature (Colt and Tchobanoglous, 1978) and its ubiquity in surface water systems. It is discharged in large quantities in industrial, municipal, and agricultural waste waters. Nitrite is not considered such a severe environmental problem because, although it is extremely toxic to aquatic life (eg. Tomasso and Carmichael, 1986; Hilmy et al., 1987), it does not usually occur in natural surface water systems at concentrations considered deleterious to aquatic organisms. Although nitrate can be found in relatively high concentrations in surface waters, it poses a less serious environmental problem because it is relatively nontoxic to aquatic organisms.

The three nitrogenous compounds are interrelated through the process of nitrification in the nitrogen cycle (Fig. 2.1). During nitrification, ammonia is oxidized to nitrate in a two-step process by two groups of aerobic chemoautotrophic bacteria (Stanier et al., 1970) and nitrite is produced as an intermediate product. The nitrification of ammonia can be summarized by the following steps:

$$2NH_3 + 3O_2 \xrightarrow{\text{Nitrosomonas}} 2NO_2^- + 2H^+ + 2H_2O$$

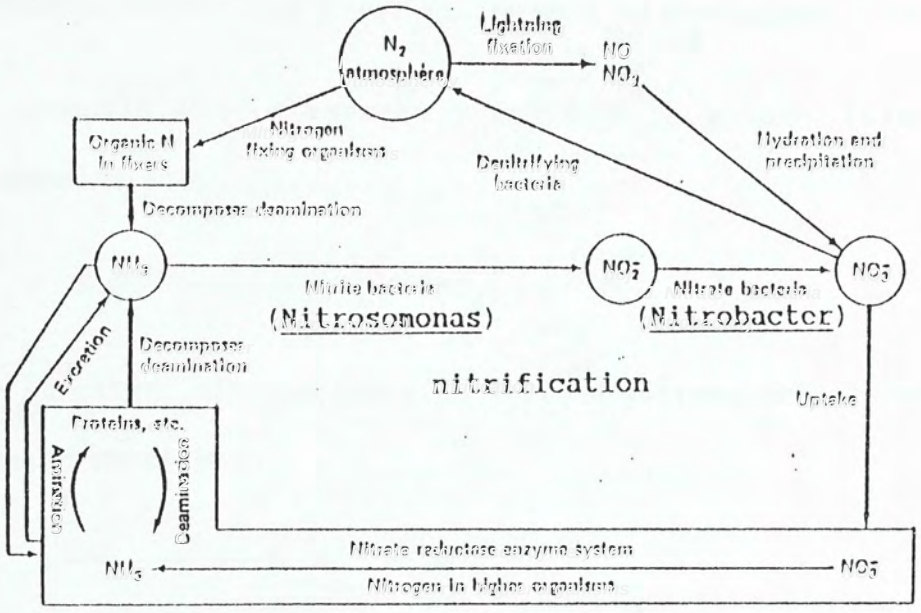

Figure 2.1. Major processes of the nitrogen cycle within the biosphere. Circular figures indicate abiotic environmental pools, rectangular figures biotic pools.

Adapted from Collier et al. (1973).

$$2NO_2^- + O_2 \xrightarrow{\text{Nitrobacter}} 2NO_3^-$$

In relatively stable oxygenated natural water systems the oxidation of nitrite to nitrate is rapid, but the conversion of ammonia to nitrite is the rate limiting step in the overall process. These chemical species can exist in ionized or un-ionized forms.

Ammonia gas is extremely soluble in water, forming the ionized ammonium ion.

$$NH_3 + H_2O \longleftrightarrow NH_4^+ + OH^-$$

In water, nitrite forms un-ionized nitrous acid according to the following equation:

$$HNO_2 \longleftrightarrow H^+ + NO_2^-$$

2.2. ACCUMULATION OF NITRITE IN NATURAL WATER SYSTEMS

Nitrite is a naturally occurring anion in fresh and marine waters. Its concentration in oxygenated waters is typically less than 0.005 mgl^{-1} NO_2-N (Lewis and Morris, 1986). Under some circumstances, however, concentrations of nitrite may be sufficiently high to be toxic to aquatic organisms.

Two genera of bacteria Nitrosomonas and Nitrobacter are involved in the nitrification process and these two genera have different sensitivities to various environmental factors. Therefore, factors that affect these two genera differentially may lead to accumulation of nitrite. For example, Nitrobacter spp. is more sensitive to un-ionized ammonia than Nitrosomonas spp. Un-ionized ammonia inhibits the activity of nitrobacters at concentrations (0.1-1.0 mgl^{-1} NH_3) appreciably lower than those (10-150 mgl^{-1}) that inhibit nitrosomonads (Anthonisen et al., 1976). Acidity affects the amount of un-ionized ammonia. Increase in pH results in increase in concentration of un-ionized ammonia which, in turn, inhibit nitrate formation and cause accumulation of nitrite. Other factors that differentially affect these bacteria can also cause accumulation of nitrite.

Certain human activities increase the amount of nitrite in aquatic systems. Several organic compounds found in industrial effluents were shown to inhibit the nitrification process. Hockenbury

and Grady (1977) found that dodecyclamine, aniline, and n-methylaniline at concentrations less than 1 mgl^{-1} caused 50 % inhibition of ammonia oxidation by Nitrosomonas; p-nitrobenzaldehyde, p-nitroaniline, and n-methylaniline at 100 mgl^{-1} inhibited nitrite oxidation by Nitrobacter. Loss of nitrification flora, resulting from use of antibiotics, has also been mentioned (Patrick et al., 1979) as a potential cause of large amounts of nitrite accumulating in natural waters. Sewage effluents can contain high amounts of nitrite (Anthonisen et al., 1976). Nitrite can also be produced in large quantities by some types of aquaculture (Schwedler and Tucker, 1983). Furthermore, fish culture facilities using closed water reuse system generally employ the nitrification process to reduce concentrations of ammonia, the primary nitrogenous waste of fishes. Effectiveness of the conversion process depends greatly on balances in the relative abundance of nitrifying bacteria and some environmental factors including pH, temperature, dissolved oxygen and concentrations of inhibiting compounds. Unfavorable conditions may lead to imbalances in the relative abundance of Nitrosomonas and Nitrobacter, resulting in elevated nitrite levels in aquacultural systems. The amount of nitrite discharged may raise the concentration of nitrite in the receiving water to levels toxic to aquatic organisms.

In confined bodies of water such as lakes or ponds, large quantities of nitrite can also accumulate due to the contamination by runoff from surrounding land fertilized by nitrate and ammonia.

Nitrite may also accumulate in the deep layers of lakes during stratification and subsequently appear at the lake surface when the lake mixes (Infante et al., 1979; cited in Lewis and Morris, 1986).

Nitrite may thus be present at unusually high concentrations under some circumstances in natural waters. Some field data are available on environmental concentrations of nitrite. McCoy (1972) found concentrations up to 73 mgl^{-1} NO_2-N in Wisconsin lakes and streams. Kingler (1957) reported nitrite concentrations > 30 mgl^{-1} NO_2-N in waters receiving effluents from metal, dye and celluloid industries. During routine analysis of water quality in a 1300-l tank employing closed seawater system for the culture of Lates calcarifer with a stocking density of 1.31 gl^{-1}, Woo et al. (1988) found concentrations of nitrite rising from a negligible level to 8 mgl^{-1} NO_2-N within 8 days.

2.3. NITRITE UPTAKE

2.3.1. CHLORIDE CELLS

Based on the morphological description of gill epithelia in fish by Laurent and Dunel (1980), two main types of epithelia are encountered in fish gill: the primary and the secondary gill epithelia. The primary epithelium covers the primary lamellae including the interlamellar region and the secondary epithelium covers the free part of the secondary lamellae. Chloride cells are specialized ovoid-shaped cells that are located on the gill epithelia and are responsible for ion regulation. Chloride cells are present in both euryhaline and stenohaline species of freshwater or saltwater fishes and exhibit specific significant characteristics in relation with the respective milieu. The ultrastructural characteristics of chloride cells are well studied (Doyle and Gorecki, 1961; Kikuchi, 1977; Pisam, 1981). Briefly, these cells display numerous mitochondria associated with a densely branched tubular system that opens on the basolateral plasma membrane. The apex of each cell has microvilli and is firmly bound to the neighbouring pavement cells by a long and tight junctional apparatus. Vesicles of various size are concentrated in the apical region.

In marine species or seawater-adapted euryhaline species, the distribution of chloride cells is usually limited to the primary epithelium. Numerous studies have provided evidence for the view that

the seawater chloride cell is involved in active excretion of unwanted ions in a seawater environment (eg. Doyle and Gorecke, 1961; Maetz and Bornancin, 1975; Foskett et al., 1983).

In freshwater species, numerous chloride cells can be found over almost all of the gill epithelia, particularly the secondary epithelium. It has been shown that rainbow trout responds to a hypotonic environment by producing chloride cells on the secondary lamellae (Krous et al., 1982). Conditions that would tend to exaggerate ion losses (such as skin wounds, external fungal infections, or the transfer of fish from fresh to deionized water) have been shown to stimulate proliferation of lamellar chloride cells (Laurent and Dunel, 1980). In addition, Girard and Payan (1980) have demonstrated that in freshwater trout, $^{24}Na^{+}$ and $^{36}Cl^{-}$ enter the gill only through the secondary lamellae. Therefore, the fresh water chloride cells are believed to function in the inward transport of ions in dilute media. In fresh water, the chloride cells can give off ammonium or hydrogen ions in exchange for equal number of sodium ions and can give off bicarbonate ions in exchange for an equivalent number of chloride ions.

2.3.2. NITRITE UPTAKE IN FRESHWATER FISH

Nitrite is a serious toxicant to freshwater fish even when it is present in the environment in a relatively low concentration because nitrite can rapidly move from the water into the organism, so

reaching surprisingly high concentrations in blood and tissues (Margiocco et al., 1983). Most freshwater fish take up nitrite against a concentration gradient; nitrite concentrations in the blood or blood plasma may reach 10 times the concentrations in the surrounding medium (Bath and Eddy, 1980). Margiocco et al. (1983) even found up to 60 times magnification of nitrite in blood, 30 times in brain and liver of rainbow trout exposed to nitrite in fresh water.

An early explanation for nitrite uptake against a concentration gradient has been offered by Colt and Tchbanoglous (1976) and Wedemeyer and Yasutake (1978). They proposed a passive diffusion mechanism whereby nitrite diffuses across gill epithelium as lipid-soluble nitrous acid (HNO_2). As mentioned in Section 2.1, some nitrite in water can combine with hydrogen ions to form nitrous acid. Within the pH range of most natural waters, the nitrite ion is 4 to 5 orders of magnitude more concentrated than the nitrous acid with which it is in equilibrium. Because nitrous acid has no electrical charge, it would not be pumped into the body fluids through the chloride cells, but it is soluble in lipids (Munn and Allen, 1974) and therefore might enter the gills through epithelial cells. Inside the fish, nitrous acid dissociates to nitrite and hydrogen ions. When the level of nitrite ions in the plasma exceeds that in the environment, the osmotic gradient reverses and nitrite ions diffuse back across the gill epithelia to the environment. However, nitrous acid can diffuse into the fish at a rate faster than nitrite can diffuse out.

Moreover, the nitrite ions inside the fish can be retained efficiently by physiological mechanisms that prevent loss of ions. For example, hemoglobin acts as a sink for nitrite ions, as is the case for oxygen. According to this explanation, the build up of nitrite would be especially pronounced when the pH of the fish (about 8.0 at the gills) is higher than that of the surrounding medium.

Both nitrite toxicity and blood accumulation are known to be lowered by environmental chloride and by inhibitors of chloride uptake (Bath and Eddy, 1980). For this reason, it has also been suggested that nitrite ions may be pumped across the gill epithelia of freshwater fish by the anion exchange mechanism that are primarily arranged for active chloride uptake in the chloride cells (Bath and Eddy, 1980; Meade and Perrone, 1980; Krous et al., 1982; Eddy et al., 1983). This hypothesis explains the strong bioaccumulation characteristic of nitrite because it is presumed that nitrite is involved in a pump mechanism arranged for a physiological ion uptake (Gaino et al., 1983). If nitrite ion and chloride ion compete for the same site on the uptake mechanism in the freshwater chloride cells, then in the absence of chloride ion or at low chloride concentration, nitrite uptake will predominate and approach the rate at which chloride would normally be transported. And this has been proved by Eddy et al. (1983) who found that the rate of nitrite uptake appeared to be very similar to the rate of chloride uptake. Krous et al. (1982) have demonstrated a direct correlation between plasma nitrite

level and the number of lamellar chloride cell in rainbow trout. Presence of nitrite in fresh water causes enlargement and rapid turnover of chloride cells (Gaino et al., 1984). The fish apparently maintain a fixed internal chloride concentration even when nitrite is present in quantities that markedly inhibit the gill carbonic anhydrase activity; this may cause the chloride cells to do more work when nitrite is present.

The literature, although not conclusive on the role of nitrous acid, does strongly support the idea that the nitrite ion enters fish through the chloride cells (Tomasso et al., 1980; Kreus et al., 1982). The recent discovery by Palachek and Tomasso (1984b) that the largemouth bass, unlike other freshwater fishes, never had plasma nitrite concentrations higher than those in test water is a particularly persuasive argument against the theory of passive nitrite accumulation through nitrous acid penetration of the gills. The additional significance of nitrous acid has to do largely with the importance of environmental pH as a regulator of nitrite toxicity. If nitrite enters fish primarily as nitrous acid, the relative internal and external pH levels are of great importance. If nitrite enters as ions primarily through the chloride cells, then the expected role of pH is considerably smaller. This matter will be considered below in connection with pH.

2.3.3. NITRITE UPTAKE IN SEAWATER FISH

The response of seawater fish to nitrite has not been investigated in detail and the amount of published information concerning how nitrite enters the seawater fish is very limited. Studies so far revealed that seawater fish or seawater-adapted euryhaline fish are less susceptible to nitrite toxicity and never had plasma nitrite concentrations higher than those in test water as did their freshwater counterparts (Meade and Perrone, 1980; Eddy et al., 1983; Scarano et al., 1984). The high chloride concentrations in sea water should competitively inhibit nitrite uptake in fish exposed to nitrite in sea water. Eddy et al. (1983) suggested that nitrite entry to the blood of seawater fish is by passive diffusion which would continue until equilibrium except that a lethal blood concentration is first achieved. Since the pH values of sea water and blood are more similar, then at equilibrium HNO_2 will exist in approximately equal concentrations on either side of the gills and the diffusion mechanism described for freshwater, if operating, would not lead to large NO_2 concentration gradients between blood and water.

In conclusion, the passive diffusion mechanism, though it remains a simple and attractive theory, it does not account for the protective effect of environmental chloride ions. The active pumping mechanism which has stronger experimental basis seems to outweigh the diffusion mechanism to account for the nitrite uptake in fish body. However, it does not rule out the possibility that both mechanisms are

operating at the same time to transport nitrite into the fish.

2.4. RESPONSES INDUCED BY NITRITE TOXICITY IN FISH

2.4.1. HEMATOLOGICAL RESPONSES

2.4.1.1. CHANGES IN METHEMOGLOBIN CONTENT

Although nitrite may have multiple modes of toxicity in fish, the best investigated and thus understood is concerned with hematology. Nitrite, being an oxidant, can diffuse into red blood cells where it oxidizes hemoglobin to methemoglobin (ferrihemoglobin) which lacks the capacity to bind oxygen reversibly (Bodansky, 1951) and thus impairs oxygen transport by blood.

The capacity of hemoglobin to bind and transport oxygen greatly depends on the presence of a heme group which contains an iron atom in the centre of the protoporphyrin ring (Fig. 2.2). The heme iron in hemoglobin is present in the ferrous (Fe^{2+}) state. Hemoglobin combines loosely with oxygen to form the easily dissociated compound oxyhemoglobin, in which the iron atom is still in the Fe^{2+} state. Nitrite oxidizes the iron in hemoglobin to the ferric (Fe^{3+}) oxidation state to form methemoglobin. Methemoglobin unlike oxyhemoglobin is a true oxidation product of hemoglobin and is therefore incapable of transporting oxygen. Because it is unable to act as an oxygen carrier, methemoglobin in significantly high concentrations will seriously reduce the total oxygen carrying capacity of the blood and cause hypoxia (Cameron, 1971a). Methemoglobin gives the whole blood a characteristic brownish colour.

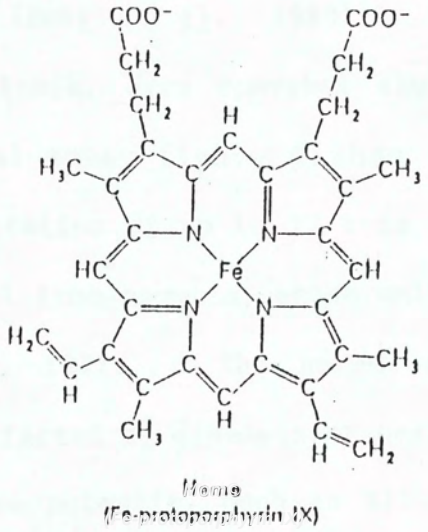

Figure 2.2. Structure of the heme group.

Adapted from Stryer (1981).

Methemoglobin forms spontaneously in normal erythrocytes, although slowly, in the absence of nitrite. Thus fish blood typically contains a measurable amount of methemoglobin even in the absence of nitrite: reported values include 0.9 to 3.6 % for rainbow trout (Cameron, 1971a; Brown and McLeay, 1975; Smith and Russo, 1975), 10.9 % for prespawning pink salmon (Cameron, 1971a) and 2.2 % for channel catfish (Huey et al., 1980). It is evident that the presence of methemoglobin, even somewhat above 10 %, should not be viewed as exceptional among fish. Graham (1986) reported a high methemoglobin concentration of up to 27 % in the Atlantic cod. Fish differ in this respect from mammals, whose methemoglobin levels seldom exceed 1 % (Cameron, 1971a). The normal methemoglobin-hemoglobin equilibrium can be affected by diseases or certain chemicals with high oxidative or reductive potential such as nitrite, ascorbic acid, and methylene blue. Factors affecting the state of energy metabolism of the red cells such as overall nutrition, hypoxia, exercise or erythrocyte age are also suggested to affect the equilibrium point between hemoglobin and methemoglobin in fish (Cameron, 1971a). Methemoglobin levels in some fishes such as cunner show significant seasonal variation (Graham et al., 1986).

Most fish species form methemoglobin readily if exposed to nitrite in waters of low chloride content (eg. Brown and McLeay, 1975; Huey et al., 1980; Tucker and Schwedler, 1983; Bartlett et al., 1987; Hilmy et al., 1987). One exception is the largemouth bass,

which forms large amounts of methemoglobin only in response to very high nitrite concentrations (Palachek and Tomasso, 1984b). Percent methemoglobin can be predicted by exposed nitrite concentration. For example, Watenpaugh et al. (1985) reported that the percent methemoglobin in treated channel catfish was highly and significantly correlated with nitrite concentration by % methemoglobin = 5.77 + 46.77 NO_2^- (r^2 = 0.72, P<0.001).

When the methemoglobin content of blood exceeds 70 to 80 % of the total hemoglobin, most fish species become torpid. This behavioural response has been documented for European minnow (Klingler, 1975), for chinook salmon (Westin, 1974), and for channel catfish (Konikoff, 1975). As the methemoglobin content of the blood approaches 100 %, fish typically become unresponsive and disoriented.

The actual stress of an equal amount of methemoglobin in an individual fish depends on many factors including behaviour, physical conditions and environmental conditions. A fish exhibiting dominant behaviour and a high degree of activity would generally have a higher requirement for oxygen, possibly resulting in a decreased tolerance to high levels of methemoglobin (Smith and Williams, 1974). Inactive fish have a very low oxygen demand and thus may not be immediately threatened by severe methemoglobinemia (Crawford and Allen, 1977). However, if a fish with elevated methemoglobin is frightened, or is otherwise forced to become active, it may die of anoxia (Huey et al., 1980). Fish in good physical condition may be able to derive a

greater portion of their energy requirements anaerobically from a high supply of stored liver glycogen. This may enable them to cope with elevated levels of methemoglobin for longer periods by lowering their requirement for oxygen. The nature of the bioassay system used (flow-through or static), the method and extent of aeration, the concentration of un-ionized ammonia and dissolved oxygen level could also exert a limiting effect on methemoglobin tolerances (Perrone and Meade, 1977).

As mentioned above, the amount of methemoglobin necessary to kill, to reduce growth of, or to induce abnormal behaviour varies with the species and with the environmental conditions. As a rough rule of thumb, methemoglobin concentrations in excess of 50 % could be considered threatening to fish (e.g. Bowser et al., 1983), although healthy fish have been taken from nature under circumstances leading to methemoglobin concentrations above 50 % (eg. Schwedler and Tucker, 1983). Channel catfish with 100 % methemoglobin have survived for 2 days in warm water (25^{o} C), although the fish were inactive (Tomasso et al., 1979). When methemoglobin concentrations are below 50 %, there is usually no mortality.

There exists some protective mechanisms which can reduce methemoglobin back to hemoglobin. Scott et al. (1965) have demonstrated that in human erythrocytes, reduction of methemoglobin is accomplished principally by the enzyme NADH-methemoglobin reductase (diaphorase I), although reduced glutathione, ascorbic acid and NADPH-

methemoglobin dehydrogenase are all able to reduce methemoglobin to a lesser extent. In red blood cells of fish, only NADH-methemoglobin reductase system has been reported to reconvert methemoglobin to hemoglobin (Cameron, 1971a; Huey and Beitinger, 1982; Freeman et al., 1982). NADH-methemoglobin reductase system is present in a variety of fish. Out of thirteen phylogenetically diverse piscine species that have been tested by Freeman et al. (1982), ten species demonstrate the NADH-methemoglobin reductase activity. This enzyme system will typically restore the normal proportion of hemoglobin within 24-48 hours if a fish is transferred to water that lacks nitrite (Huey et al., 1980). When nitrite is present, the ultimate level of methemoglobin in the blood is a result of the balance between methemoglobin formation and reconversion to hemoglobin by the reductase.

2.4.1.2. CHANGES IN TOTAL HEMOGLOBIN CONTENT, ERYTHROCYTE COUNT AND HEMATOCRIT

Literature concerning changes in total hemoglobin content caused by nitrite exposure is rather inconsistent. A significant reduction in total hemoglobin has been reported for rainbow trout in fresh water after 96 hour exposure to 0.1 to 0.5 mgl^{-1} NO_2-N (Brown and McLeay, 1975; Margiocco et al., 1983), and for channel catfish in fresh water after 96 hour exposure to 28-32 mgl^{-1} NO_2-N and an extended period of 6 month exposure to 2.8-3.2 mgl^{-1} NO_2-N (Hilmy et al., 1987). Hemoglobin decrease was observed in largemouth bass

exposed to 150 mgl^{-1} NO_2-N for 96 hours in sea water but such decrease was not observed in the first 24 hour exposure (Scarano et al., 1984). On the other hand, hemoglobin reduction was not observed in rainbow trout after 6 month exposure to 0.015-0.060 mgl^{-1} NO_2-N (Wedemeyer and Yausutake, 1978), and was not reported in channel catfish exposed 24 hours to 5 mgl^{-1} NO_2-N (Huey et al., 1980), nor in bullfrog (Rana catesbeiana) tadpoles exposed 24 hours to 1.0-50 mgl^{-1} NO_2-N (Huey and Beitinger, 1980a). To sum it up, nitrite exposure appears to be able to reduce total hemoglobin content on condition that exposure time is sufficiently long or exposure concentration is sufficiently high. Decreases in hematocrit (Eddy et al., 1983; Wilmy et al., 1987) and erythrocyte count (Wilmy et al., 1987) have also been reported in fish exposed to nitrite.

Nitrite exposure definitely causes reduction in functional hemoglobin as a result of a combination of methemoglobin increase and total hemoglobin decrease. This may seriously impair the oxygen transport capacity of blood and lead to hypoxia (Cameron, 1971a). Previous papers (eg. Smit and Wattingh, 1978b) have stated that hemoglobin and hematocrit values were increased as compensation for hypoxia and such increases were achieved by hemoconcentration and swelling of the red blood cells (Albers, 1970). It appears likely that the decrease in the values of three hematological parameters observed during nitrite exposure is due to the toxic effects of nitrite rather than hypoxia. Scarano et al. (1984) suggested that

during nitrite exposure, an adaptive mechanism occurred in red blood cells of the fish. At nitrite concentrations high enough to induce methemoglobinemia, metabolic energy would be utilized by erythrocytes for methemoglobin reduction. Such a mechanism would reduce the half-life of the red blood cell pool. Red blood cells would probably be destroyed by spleen and kidney macrophages. Histochemical analysis of spleens from treated fish really demonstrated that macrophages were engulfing red blood cells (Scarano et al., 1984).

2.4.2. BIOCHEMICAL RESPONSES

2.4.2.1. CHANGES IN PLASMA OR SERUM ION CONCENTRATION

Changes in plasma or serum ion concentration in fish exposed to nitrite can more or less give some idea on how nitrite intoxication affects the osmoregulation of fish. Some studies in this area reported that no significant changes were observed in concentrations of plasma sodium (Bath and Eddy, 1980), plasma chloride (Bath and Eddy, 1980) and blood chloride (Gaino et al.; Margiocco et al., 1983) in nitrite-exposed fish. No work has been done on the effect of nitrite on other serum or plasma ion concentration such as potassium and calcium.

2.4.2.2. CHANGES IN BLOOD AND TISSUE METABOLITE CONTENTS

There are only a very limited number of studies that deal with the biochemical responses of nitrite-exposed fish. An early study by Colt et al. (1981) on channel catfish that had been held for 31 days at sublethal nitrite concentrations suggested that nitrite did not have a significant effect on water content of the fish.

One detailed study was carried out by Arillo et al. (1984) who investigated the main biochemical end-products of cerebral and hepatic hypoxia in rainbow trout specimens exposed for 12, 24, 48, and 72 hours to nitrite (0.45 mgl^{-1} NO_2-N). They found that lactate, glycerophosphate, and succinate increased in the liver of nitrite-

treated trouts; such increases reached high statistical significance at different times from the beginning of the experiment: 72 and 24 hours for lactate and glycerophosphate respectively, while succinate increase was statisically significant only in overturning trout. ATP and sugars also decreased significantly in the treatment groups. Lactate was significantly elevated in brains of overturned trout, neither α-glycerophosphate nor succinate showed any significant changes. Sugars and ATP in the brain remained stable in even torpid fish and decreased only in overturned ones. Blood lactate showed a significant rise in torpid trout exposed 72 hours as well as in all overturned fish, while blood glucose was lower only in overturned fish. Lactate, glycerophosphate, glycogen, ATP and succinate are biochemical indicators of hypoxia. Biochemical responses to hypoxia or anoxia are well studied in several systematic groups including fish (Jorgensen and Mustafa, 1980a, b). It is assumed that when glycogen and ATP decrease, and at the same time there is an increase in lactate, α-glycerophosphate, and succinate, an hypoxic (anoxic) situation is occurring. On this basis, it is clear that the liver of nitrite-treated trout shows a slightly hypoxic situation. In more advanced stages of intoxication such as in these trout treated for as long as 72 hours, the hypoxic situation becomes more typical. Only in overturning specimens are biochemical data so altered as to indicate a severe hypoxic state: here, in addition, succinate increases, so illustrating an impairment of mitochondrial function. It can therfore be concluded that, except for overturning animals, serious biochemical

symptoms of hypoxic damage are not detectable, even in those trouts which are in very bad physiological condition and which otherwise show signs of intoxication. The results also show clearly that in the brain of normally swimming fish no significant symptom of hypoxia can be found, and even in overturned specimens the hypoxic state is weak and incomplete.

In another study, a decline in serum total protein levels was recorded during acute and chronic exposure of Clarias lazera to nitrite (Hilmy et al., 1987). Newcomb (1974) reported a similar decrease in serum protein concentration in juvenile steelhead trout exposed to nitrogen supersaturation for 35 days. Besides, a great variety of systemic stressors including toxicants also tend to diminish the total blood protein, although these values recover during the stage of resistance to stress (Selye, 1950). Therefore, the decline in serum total protein may be a generalized stress response or a result of hypoxia caused by methemoglobinemia.

2.4.2.3. ALTERATIONS IN MITOCHONDRIAL FUNCTION

Arillo et al. (1984) have carried out in vitro investigations on liver mitochondria incubated in a medium containing 15 $\mu g ml^{-1}$ NO_2-N, which is a nitrite concentration similar to that found in the liver of rainbow trout treated in vivo to 0.45 $mg l^{-1}$ NO_2-N. It was found that nitrite could markedly impair mitochondrial function, respiratory control ratio (RCR) and oxygen consumption

showed a relevant decrease when the toxicant is added, at least after a 10-min preincubation. The decrease of index RCR was due both to an activation in state 4 and to a decrease in state 3 oxygen consumption as reported for mammalian mitochondria (Inoue, 1979). The modification in state 4 might be interpreted as a consequence of some membrane alteration, leading to an uncoupling-like effect. The decrease in state 3 was probably due to a nitrite-induced inhibition of cytochrome C oxidase, as reported for rat skeletal muscle mitochondria (Walter and Taylor, 1965). Such an impairment of mitochondrial function has an early *in vivo* effect on the liver ATP stores. *In vivo* experiments carried out in the same study revealed that the liver ATP concentration in treated specimens began to decrease already after 12 hours treatment while the changes of other biochemical parameters are much more moderate.

2.4.2.4. ALTERATIONS IN LYSOSOMAL FUNCTION

Recent investigation revealed that lysosomes are good markers of stress. It is known that a variety of environmental stressors can bring about alterations in lysosomal function. Some chemical properties of nitrite suggest that it may affect lysosomal behaviour. Nitrite is known to mediate, through some of its products (nitrous acid and nitric oxide), the binding of thiol and amino groups (Knowles *et al.*, 1974; Ignarro and Gruetter, 1980), thus inducing selective chemical modifications in both functional and structural

proteins. As a consequence, changes in the function of enzymes and in the permeability of the lysosomal membrane may be expected. Mensi et al. (1982) examined some parameters of liver lysosomal function in rainbow trout exposed to 0.45 mgl^{-1} NO_2-N (nearly 36 hr LC50) for various exposure times (12-72 hours). In vivo both total proteolytic activity and single protease activity of cathepsin B, cathepsin C, and leucylaminopeptidase of liver were found to be inhibited , the inhibition increasing with the lengthening of exposure time. In vitro no analogous effect was observed. These findings indicated that the decrease in protease activity found in vivo is not primarily due to a direct interaction with nitrite. Since the inhibition observed in treated trout seemed to be proportional to the exposure time, the authors suggested that nitrite caused an indirect time-dependent impairment in protease synthesis. The mechanism that leads to this impairment is unknown at present. However, since nitrite brings about a relatively time-dependent decrease in ATP levels (Arillo et al., 1984), it is reasonable that the energy-dependent synthesis of lysosomal hydrolases is altered thus producing structurally inadequate and consequently inactive enzymes. However, it does not rule out the possibility that nitrite products, such as nitric oxide, may disturb hydrolase synthesis per se by interfering in some steps in its synthesis.

Another finding was that lysosomal membranes showed an increased fragility in treated specimens. It was demonstrated that

nitrite affected membrane stability both by worsening the sensitivity to osmotic shock and causing per se the rupture of organelles in iso-osmotic suspension. Since results concerning lysosomal lability were in line with the pattern followed by hepatic nitrite concentration, the author suggested that nitrite directly affected lysosomal integrity probably through the membrane structural modifications which the toxin is able to mediate. In fact the interaction of amino and thiol groups of membrane proteins with nitrous acid or nitric oxide lead to alteration in the structure of proteins, thus varying the integrity and the permeability properties of the membrane. Moreover, nitrite has been shown to mediate lipid oxidation through its product NO_2 (Estefan et al., 1970). Therefore, the membrane may be further damaged because of alterations in its phospholipidic components.

2.4.2.5. CHANGES IN ENZYME ACTIVITIES

Nitrite was shown to markedly inhibit the activity of branchial carbonic anhydrase in vitro in concentrations (5-40 ugl^{-1} NO_2-N) similar to those determined in various tissues of in vivo treated rainbow trout (Gaino et al., 1984). The inhibition of carbonic anhydrase should impair the osmoregulatory function of chloride cells because of the decreased production of bicarbonate (HCO_3^-), which is believed to exchange for chloride (Maetz and Garcia-Romeu, 1964). There is no study concerning the effects of nitrite on the activities of other branchial enzymes such as Na^+-K^+-ATPase.

Michael et al. (1986) observed an increase in serum glutamate-oxaloacetate (SGOT) and glutamate-pyruvate transaminases (SGPT) activity in two sizes of juvenile Clarias lazera during chronic exposure (6 months) to 2.8 and 3.2 mgl^{-1} NO_2-N, which were one tenth of the 96 hr LC50 values for the two size respectively. And there is a good correlation between the rise in these enzyme activities and the severity of histological damage. SGOT and SGPT have been reported to be responsive to liver injury in man and animals. Therefore, the increases in both enzyme activities during nitrite exposure indicate that nitrite can induce hepatic damages in fish.

In vitro study on the effect of nitrite on lactate dehydrogenase and glucose-6-phosphate dehydrogenase was carried out by Arillo et al. (1984) who found that both enzymes did not undergo any change in their activities even in the presence of high nitrite concentrations; a long incubation of the extract in the presence of nitrite concentrations four to five times higher than those detected in the liver of the fish exposed in vivo caused no notable changes in enzyme activities.

2.4.3. ULTRASTRUCTURAL RESPONSES

Wedemeyer and Yasutake (1978) examined tissues of rainbow trout that had been held for 28 weeks at sublethal nitrite concentrations. The kidneys, blood, and thymus did not show damage; slight changes in the gill tissues including minimal hypertrophy, hyperplasia, and lamellar separation, judged by the authors to be of minimal importance, were observed after 3 weeks but had disappeared after the fish had been held 7 weeks. After 28 week exposure most of the test fish had recovered and showed little or no lamella epithelial change. Colt et al. (1981) found no gill damage in channel catfish, even at lethal concentrations of nitrite. Also Michael et al. (1986) reported that kidneys from fish exposed to nitrite were not noticeably different histologically from that of control fish. On the contrary, there are studies demonstrating that nitrite can induce histological changes in liver and gill tissues of fish.

Krous et al. (1982) found that the numbers of lamellar chloride cells were directly correlated with plasma nitrite values in rainbow trout. Moreover, Gaino et al. (1983) reported that nitrite induced an acceleration of chloride cell turnover both producing hyperactivity in some cells enhancing their degenerating rates in rainbow trout. The gills of treated specimens, in comparison with controls, had a great number of chloride cells at all stages of development (active, degenerating and completely degenerated cells). In particular, fully active cells, with intact cytoplasm, often became

so hypertrophic as to completely project outside the epithelium: this led to an enhancement of the total surface area and probably to a greater absorption of dilute electrolytes from the surrounding medium. In the same study, the authors also found that in the blood of the treated trout the chloride concentration remained constant in spite of a considerable uptake of nitrite. In vitro observation also showed that nitrite could markedly inhibit the activity of carbonic anhydrase. Therefore, the hypertrophia in some gill chloride cells could be a hyperactive response attempting to maintain physiological chloride levels in the presence of nitrite and lowered bicarbonate production. Histopathological study of gills of Clarias lazera exposed chronically for six months to sublethal nitrite concentration by Michael et al. (1986) found that nitrite exposure induced lesions such as hypertrophy and hperplasia in the epithelial surface of the respiratory lamellae. Lifting of epithelial cells was also prominent. Necrotic respiratory lamellar epithelium was observed during the fourth, fifth and sixth months.

Hypertrophy, hyperplasia and lamellar separation of gill epithelium is commonly seen in fish exposed to almost any irritant. For example, gill hyperplasia was observed in salmonids exposed to ammonia (Larmoyeux and Piper, 1973). Thurston et al. (1978) found that gills of cutthroat trout fry exhibited hypertrophy of lamellar epithelium, some necrosis of epithelial cells and separation of epithelium as a result of treatment with ammonia for 29 days. Abdel-

Aziz (1982) recorded hyperplasia and hypertrophy in lamellar epithelia of Clarias lazera and Tilapia zilli following acute exposure to zinc. Swelling, hyperplasia and lifting of lamellar epithelium can serve a defense function (Mallatt, 1985) as these alterations increase the distance across which the toxicant must diffuse to reach the blood stream. Mallatt (1985) has statistically reviewed fish gill structural changes induced by toxicants and other irritants and he concludes that irritant-induced gill alterations do not reflect specific actions of irritants as much as they seem to reflect physiological responses of fish. In addition to the specific physiological responses of an organism to a stressor, a series of nonspecific physiological responses referred to as the general adaptation syndrome takes place (Selye, 1950). Part of the syndrome is the release of corticosteroid hormones from the interrenal glandular tissue. Scott and Rogers (1980) viewed irritant-induced gill alterations as part of the general systemic response to stress (general adaptation syndrome). Therefore, it may be concluded that the observed alterations in gill lamellae, due to nitrite exposure, can be considered as a defense response which may be partly brought about by the release of corticosteroids into circulation. However, research is needed in this area to know the extent to which these hormones may affect gill structure (Michael et al., 1986).

Arillo et al. (1984) made observations on liver ultrastructure of rainbow trout exposed acutely to nitrite and they found that mitochondrial damage was the most conspicuous of the

hepatic ultrastructural effects. After 48 hour nitrite treatment the major ultrastructural change in the liver consisted of a mitochondrial vacuolization, which could be considered a morphological expression of hypofunction of these organelles. The glycogen tended to accumulate near a pole of the cell rather than to disperse among the membranes of the smooth endoplasmic reticulum, as it did in controls. The mitochondrial situation got worse at 72 hour when the myelinic degeneration appeared, in addition to vacuolization. In overturned specimens hepatocytes showed a complete and clearly irreversible destruction of the cellular structure. Glycogen decreased dramatically and even disappeared. Mitochondria appeared to degenerate so that very large vacuoles could be observed in the hepatocytes of near-death trout. The development of the ultrastructural changes in mitochondria as well as the modifications in the glycogen granule arrangement were in line with the progressive reduction of hepatic ATP and total sugar, and with the modifications of the other biochemical indicators of hypoxia detected *in vivo*. The agreement between biochemical and ultrastructural data was stressed by the remarkable nitrite-induced impairment of the mitochondrial state 3 respiration found in the author's *in vitro* experiment, and by the nitrite-induced decrease of the respiratory control ratio as described before. Nitroso compounds have been reported to induce mitochondrial swelling and elongation in rat liver hepatocytes (Rusu *et al.*, 1980). The nitrite-induced effects on mitochondrial function of rainbow trout were certainly worse than those observed in mammals and led to a

complete degeneration of mitochondria and transformation into vacuoles. Microscopic examination of the liver of Clarias lazera indicated that exposure to nitrite produced marked hepatic alterations which was characterized by vacuolation of cells around the central vein accompanied by fatty infiltration (Michael el al., 1986).

2.4.4. RESPIRATORY RESPONSES

Respirometry may provide indirect insight into the mode of toxicity of a chemical. Oxygen consumption is one of the various measures used to assess the effects of toxicants or irritants on metabolic rates of animals. The conversion of hemoglobin to methemoglobin in nitrite-exposed fish may reduce their oxygen uptake capability, and thereby depress their oxygen consumption rate and thus metabolic rate.

Waleupaugh and Beitinger (1985a) demonstrated that acute exposure of fathead minnow to nitrite reduced their weight specific oxygen consumption by about 22 %. Routine oxygen consumption was also measured in control and nitrite-treated Amazonian fish Brycon cf. melanopterum and Semaprochilodus insignis by Bartlett et al.(1987) who found that the initial rates of oxygen consumption for both control and treated fish were not significantly different. However, as the oxygen content of water was below 20 % saturation there was a marked decrease in the rate of oxygen utilization in nitrite-treated as compared with control fish.

Although routine respiration rate determinations detected a sublethal effect of nitrite on some fishes, such determinations are usually insensitive indicators of changes in metabolic rate induced by chemicals because the activity of fish is unregulated (Anderson et al., 1980). Effects of chemicals on metabolic rate of organisms are

better evaluated with standard or active weight specific oxygen consumption when possible.

Oxygen affinity was measured on samples of whole blood taken from Semaprochilodus insignis 1 hour after intraperitoneal administration of 10 $mgkg^{-1}$ of sodium nitrite, when the concentration of methemoglobin was found to be about 30 % (Bartlett et al., 1987). The oxygen equilibria values found for control and nitrite-treated fish, measured at different pH revealed that there was a significant increase in the oxygen affinity of the blood of the fish with this relatively mild methemoglobinemia, with a more pronounced Bohr effect, as compared with the non-methemoglobinemic controls, which had a methemoglobin concentration of less than 5 %.

2.4.5. OTHER RESPONSES

2.4.5.1. GROWTH SUPPRESSION

Wedemeyer and Yasutake (1978) recorded no statistically significant growth suppression of steelhead during 6 month exposures to nitrite concentrations as high as 10 % of the 96 hr LC50. Working with channel catfish, Colt et al. (1981) found that the minimum amount of nitrite capable of causing detectable growth suppression over 31 days was equal to 44 % of the minimum nitrite concentration required to induce mortality. The study of Bowser et al. (1983) showed that the minimum nitrite required to cause mortality of channel catfish would equal approximately half of the 96 hr LC50, which implies that the minimum amount of nitrite capable of causing detectable growth suppression in channel catfish under the conditions studied by Colt et al. would be approximately one-fifth of the 96 hr LC50. The maximum growth suppression at such concentrations would be approximately 10 %; the maximum growth suppression actually observed by Colt et al. at any nonlethal concentration was 21 %.

2.4.5.2. TEMPERATURE TOLERANCE

Nitrite exposed fish may be more susceptible to unfavorable environmental factors such as low oxygen content and high temperature. Watenpaugh et al. (1985) showed that the critical thermal maximum (CTM) was slightly lower (35.9 ^{o}C) for channel catfish exposed to 0.43

mgl^{-1} of NO_2-N than for controls (38.0 ^{o}C). This is consistent with the concept that higher temperatures reduce the LC50 of nitrite by raising the tissue oxygen demand. Interactions among temperature, tissue hypoxia resulting from methemoglobinemia and possible changes in blood distribution to alleviate tissue hypoxia may explain the observed CTM decrease in nitrite-exposed channel catfish. It is because elevated temperature increases the metabolic rate of ectotherms and hence their oxygen demand. Methemoglobin formation most likely decreases oxygen transport to the tissue and the decreases in oxygen carrying capacity would at least contribute to reduced ability to tolerate increasing temperatures.

The lower tolerance to increasing temperature indicates that nitrite-temperature interactions have the potential of adversely affecting the productivity of high-density aquaculture of warmwater fish such as channel catfish.

2.4.5.3. SWIMMING PERFORMANCE

Several investigators have identified swimming performance as a potentially sensitive indicator of sublethal stress in fish (e g. Schneider and Connors, 1982). Farlinger and Beamish (1977) subdivided swimming performance of fish into three general categories: sustained swimming that utilizes only aerobic metabolism and can continue indefinitely, prolonged swimming that depends on both aerobic and anaerobic metabolisms and cannot be sustained indefinitely, and burst

swimming that lasts only seconds and thus is supported by anaerobic metabolism. Tests of prolonged swimming are generally considered most useful in sublethal stress assessment, as they draw on both major biochemical energy sources. Watenpaugh and Beitinger (1985b) has determined the effect of nitrite exposure on the prolonged swimming performance of channel catfish. Swimming performance was quantified as time (in min) to exhaustion in a recirculating system designed to produce current flow through the swimming channels. It was demonstrated that exposure of channel catfish to nitrite significantly reduced their prolonged swimming performance and the swim time performance was inversely correlated with percent methemoglobin. The reduction in prolonged swimming performance of nitrite-exposed channel catfish was probably due in part to methemoglobin formation. Methemoglobin in blood reduces delivery of oxygen to tissues by making hemoglobin unavailable for oxygen transport and by shifting the oxygen dissociation curve to the left (Bodansky, 1951). Such a shift increases the affinity of hemoglobin for oxygen, thereby making it less able to unload oxygen to the tissues. Channel catfish with large percentages of their hemoglobin oxidized to methemoglobin by nitrite must rely more heavily on anaerobic glycolysis than aerobic metabolism to fuel locomotion activity. Glycolysis, in addition to bearing only a short term energy source for vertebrates, produces lactate as an end product. Muscle glycolysis still eventually requires oxygen to metabolize the toxic lactate after it is transported to the liver. Reduced aerobic metabolism of channel catfish with methemoglobinemia

should limit prolonged swimming performance. In addition to methemoglobinemia, other factors such as intraspecific variation in aerobic and anaerobic capacity also influence the swimming performance of fish.

2.5. TOXICITY MECHANISMS

Studies concerning the effect of nitrite on fish have been developed considerably along the toxicological viewpoint over the past few years. However, little data are available on the toxicity mechanism of this pollutant.

As the prominent effect of nitrite to fish is the oxidation of hemoglobin to form methemoglobin and therefore, methemoglobinemia has been suggested as the primary toxicity mechanism of nitrite. Huey et al. (1980) proposed that methemoglobinemia and resulting anoxia were apparently the primary cause of death in nitrite-exposed channel catfish. However, no evidence has yet related methemoglobinemia as the direct cause of death in nitrite-exposed fish. Many observations reveal a lack of correspondence between the percent methemoglobin and mortality, or physiological condition of treated animals. Smith and Williams (1974) observed mortality of some rainbow trout with blood methemoglobin levels lower than those of other rainbow trout that survived, and this led them to suggest that the fish died from a toxic reaction to nitrite itself rather than from methemoglobinemia. Crawford and Allen (1977) observed that in sea water with added nitrite, chinook salmon had high methemoglobin levels (74 %) but very low mortality (10 %); in fresh water with added nitrite, lower methemoglobin levels (44 %) were found in salmon, but 70 % mortality occurred. They further observed that fish that died in fresh water often had red gill lamellae, rather than the brown colour typically

caused by methemoglobinemia. Almendras (1987) reported a similar result that the mortality of milk fish with the same level of methemoglobin (75 %) was about twice as great in fresh water as in 16 $^{o}/_{oo}$ brackish water. These results indicate that the toxicity of nitrite in fresh water may be attributable to some other mechanism or something in addition to methemoglobinemia. Tomasso et al. (1979) reported that channel catfish, apparently not near death, possessed very high methemoglobin content (near to 100 %). Perrone and Meade (1977) observed that generally a severe stress occurred with methemoglobin levels as high as 85 - 95 %, but some specimens could tolerate, without any apparent stress, even 89 % of methemoglobin, though showing brown blood and gill. It is suggested that such discrepancies can be due to individual differences in tolerance to anaerobic conditions; however, it also implies that some toxic mechanisms other that methemoglobinemia is associated with mortality. However, alternative mechanisms of nitrite induced mortality have as yet not been well documented.

One mechanism that nitrite induces mortality can be gill damage as gill hypertrophy, hyperplasia and lamellar separation have been reported in nitrite-exposed fish (Gaino et al., 1983; Michael et al., 1986). However, there are findings that gills of fish exposed to nitrite show either little or no damage (channel fish: Colt et al, 1981; rainbow trout: Wedemeyer and Yasutake, 1978). Smith and Williams (1974) reported changes in the thymus of rainbow trout

exposed to nitrite, but only at lethal concentrations.

Working on rainbow trout exposed to 0.45 mgl^{-1} NO_2-N for various periods of time, Margiocco et al. (1983) found that treated specimens possessed methemoglobin levels neither correlatable to their physiological conditions, nor proportional to their blood nitrite concentration. Nitrite accumulated in blood and tissues of fish to a surprisingly high extent (up to 60 times the test concentration in blood and 30 times in liver and brain) against increasing concentration gradients. In the liver and brain of treated specimens, there were great differences between apparently unstressed fish and torpid, unreactive ones. The clear correlation between physiological conditions and tissue concentration of nitrite, and the high concentration detected in the organs of moribund fish have led the authors to reach the conclusion that death following acute nitrite intoxication was probably due to nitrite toxic action in vital organs rather than to methemoglobinemia. The heavy nitrite accumulation into vital organs (liver and brain) might well result in severe metabolic damage: in fact, it is well known that nitrite strikingly reacts through its derivatives with $-NH_2$ and -SH groups, thus being able to inhibit several enzymes and to generate mutagenic compounds (Margiocco et al, 1983). Nitrite has been implicated in the formation of N-nitroso compounds (Archer et al., 1971; Wolff and Wasserman, 1972), and nitrosamines have been shown to be carcinogenic in zebrafish, rainbow trout, and guppy (Stanton, 1965; Ashley and Halver, 1968; Sato et al., 1973). Nitrite was also reported to induce cancer in

rats directly, rather than through formation of nitrosamines (Newberne, 1979).

Nitrite possesses several other toxic actions. Nitrite has been reported to induce lysosomal alterations in fish with a remarkable increase of the lability of these organelles (Mensi et al., 1982), perhaps connected with modifications of the lipids of the membrane. In mammals, for example, mitochondria and microsomes, especially when oxygen is lacking, are particularly damaged by nitrite (e.g. Walters and Taylor, 1965; Shertzer and Duthu, 1979). Arillo et al. (1984) also observed that nitrite could markedly impair mitochondrial function in rainbow trout.

As mentioned in the Section 2.4.2.2, Arillo et al. (1984) has reported that nitrite exposure does not induce significant hypoxic symptoms in liver and brain of rainbow trout. They suggested that tissue hypoxia, due to the nitrite-induced high methemoglobinemia, is too low to be directly responsible for animal death. Nevertheless liver hypoxia was thought to be at the root of nitrite acute toxicity mechanism by producing suitable conditions for toxic potentialities. The anatomic characteristics of the liver (in mammals and fish) suggest that this organ is particularly sensitive to hypoxic situations. The centrolobular area of the liver is generally in a physiological hypoxic condition (Schumacher, 1957). Nitrite-induced methemoglobinemia probably causes "local" hypoxic situations in centrolobular areas; nevertheless in early stages, such an occurrence

is not detectable through biochemical tests, which provide mean values relative to the whole organ. The "local" production of lactate together with the oxygen shortage might well create favorable conditions for nitrite to accomplish its toxic action. In fact, the chemical equilibrium between nitrite and some of its derivatives is pH regulated. It is through these derivatives that nitrite shows the most toxic effect on the cell, because it can rapidly react with lipids, in the cytoplasm as well as in the membranes, and with $-NH_2$ and -SH groups of biologically fundamental molecules (Keefer and Rolla, 1973; Ignarro and Gruetter, 1980; Knowles, 1974; Estefan et al., 1970). While oxygen tension is decreasing (perhaps methemoglobin level reaches 80 %), new toxic mechanisms occur. Generally, hemoproteins and cytochromes show more affinity for oxygen than for nitrite; but when oxygen falls below certain values, nitrite can act directly on mitochondrial cytochrome C and on microsomal P450 (Wolff and Wasserman, 1972; Walter and Taylor, 1969) thus impairing mitochondrial and microsomal functions. It is probably that at this point, the severe hypoxic state becomes evident in the overturning condition. Thus the irreversible and deadly hepatotoxic damages, particularly at the mitochondrial level may partly account for the death under nitrite intoxication.

In conclusion, the results of various studies suggest that the toxicity mechanism of nitrite can be connected with different effects on different organelles - independent or synergic- and that methemoglobinemia is only one of these.

2.6. SYMPTOMS OF NITRITE TOXICITY

Fish exposed to nitrite exhibit no unusual behaviour in the early part of exposure (Knoiff, 1975). Later they become quiet and lie motionless on the bottom of the tanks (Knoiff, 1975; Crawford and Allen, 1977; Tomasso et al, 1979). They may exhibit rapid opercular movement (Westin, 1974) and laboured respiration (Hilmy et al., 1987). Occasionally some of the fish will suddenly become active, gulp at the surface, swim in an erratic manner for a while and then die. Many of the nitrite-exposed fish show changes in body colour. For example nitrite-exposed chinook salmon changes from the normal silver-grey to almost black (Westin, 1974), while Clarias lazera changes from the normal dark grey to a pale grey colour (Hilmy et al., 1987). Overall anatomical examination of fish under nitrite intoxication reveals a distinctive brown blood and gills (e.g. Knoiff, 1975; Margiocco, 1983).

2.7. FACTORS AFFECTING THE TOXICITY OF NITRITE

2.7.1. BIOLOGICAL FACTORS

2.7.1.1. FISH SIZE

Fish size is an important consideration when evaluating nitrite toxicity data. An early study by Smith and Williams (1974) on rainbow trout showed that small fish were less sensitive to 24 hour exposures of nitrite than were larger ones. Russo et al. (1974) showed that rainbow trout larvae were slightly less susceptible to nitrite than larger fish, and subsequent studies by Russo and Thurston (1977) on rainbow trout also suggested that the larger fish are slightly more sensitive to nitrite. Thurston et al. (1978) reported no difference in the sensitivity of cutthroat trout weighing 1 and 3g. This, however, could be explained by the very small size range of the fish. For rainbow trout over the range of 2 to 387 g, Russo (1980) found no statistically significant relationship between size and mortality over 96 hours. However, Russo's data showed that the four highest LC50 values occurred in the first 5 of 20 size groups. Thus the small fish were likely to have significantly higher LC50s than the large fish.

Perrone and Meade (1977) showed that very small coho salmon (0.65 g) were less susceptible to nitrite than yearlings (22 g). In the only study demonstrating more resistance in larger fish, Wedemeyer and Yasutake (1978) concluded that rainbow trout weighing 10 g were

slightly less vulnerable to nitrite toxicity than rainbow trout weighing 5 g, although standard errors for the 96 hr LC50 values overlapped.

For warmwater fish, the only extensive study of the relationship between size and nitrite toxicity is that of Palachek and Tomasso (1984a). These authors concluded that fathead minnows weighing between 0.3 and 0.8 g were more tolerant of nitrite than fish weighing 0.9 to 3.3 g. The 96 hr LC50 for the smaller fish was about 50 % higher than that of the larger fish.

From the presently available literature it is concluded that small fish, even larvae, are unlikely to be more sensitive to nitrite than larger fish of the same species. Furthermore, there is definite evidence for some species that very small fish are less vulnerable to toxicity than fish of intermediate or larger size.

The biological basis for the size difference in tolerance is not totally understood. It has been well known that the blood of some fish species, such as European eel and Altantic salmon, show different types of hemoglobin components clearly distinct by physical, chemical and functional properties during the course of development (Rizzotti et al., 1977; Koch et al., 1966). Thus Russo et al. (1974) proposed that if rainbow trout are dying from methemoglobinemia, the higher tolerance to nitrite by young trout may be attributable to the higher oxygen affinity of the hemoglobin in larval and possibly

juvenile stages of rainbow trout when compared with adults. On the other hand, Perrone and Meade (1977) citing Kiese (1974) suggested that young individuals might have a more active methemoglobin reductase system which would counteract the methemoglobin forming tendencies of nitrite allowing fry and smaller fish to withstand increased concentration for a longer period of time.

2.7.1.2. SPECIES-SPECIFIC TOLERANCE

The literature suggests that the toxicity of nitrite varies greatly among fish species. For example, 96 hr LC50 values range from 0.27 mgl^{-1} NO_2-N for rainbow trout (Russo et al., 1974) to 140 mgl^{-1} NO_2-N for largemouth bass (Palachek and Tomasso, 1984b). There also appears to be toxicity variations within species. The reported 24 hr LC50 values for channel catfish include 10.3 mgl^{-1} NO_2-N (Konikoff, 1975), 26.2 mgl^{-1} NO_2-N (Colt and Tchobanoglous, 1976) and 1.5 mgl^{-1} NO_2-N (Tomasso et al., 1980). These interspecific and intraspecific differences are probably attributable to the different water quality characteristics that exist at the individual research facilities and to genetically based differences in nitrite susceptibility among species. The numbers and differential selectivity of chloride cells to nitrite could also account for a portion of the differences in nitrite toxicity among species.

In their literature review, Lewis and Morris (1986) summarize published nitrite bioassay data for fish and give the

experimental conditions insofar as possible. They have converted all of the LC50 data to a 96 hour basis and standardized the 96 hr LC50s to a constant chloride concentration of 20 mgl^{-1} (Table 2.3). Actually considerably more data will be required before the characteristics of individual species are known with confidence, but the summary does show evidence of much interspecific variation and some clustering of taxa. Salmonids are among the most sensitive of the taxa that have been studied, and show very little difference among species. There is considerable variation among the warmwater fish taxa: channel catfish are as sensitive as salmonids, and the logperch brook stickleback, and blue tilapia seem to be similarly sensitive or only slightly less so. The cyprinids, catostomids, mottled sculpin, and black bullhead are considerably less sensitive. The centrarchids are especially insensitive to nitrite toxicity. Critical concentrations for the largemouth bass are quite high because the blood plasma nitrite concentrations do not exceed those of the environment. Other insensitive taxa, including bluegill, mottled sculpin, catostomids, and some of the least sensitive cyprinids, should also be studied for blood plasma response to nitrite in the environment.

Table 2.3. Summary of nitrite LC50 data (median lethal concentrations) for all fish species on which there are published data

Species	Source	Cl^- (mgl^{-1})	Ca^{2+} (mgl^{-1})	Alkalinity ($CaCO_3$, mgl^{-1})	Temperature (^oC)	pH	LC50 NO_2-N	Time (hr) for LC50	LC50 adjusted to 96 hr	LC50 adjusted to 96 hr, 20 mgl^{-1} Cl^{-1}
Rainbow trout	(1)	0.35	60	176	10	7.9	0.24	96	0.24	5.90
	(2)	0.24	54	164	11	7.3	0.27	96	0.27	8.06
		0.24	54	139	12	7.2	0.15	96	0.15	7.94
		0.24	54	174	11	7.9	0.26	96	0.26	8.05
		0.24	54	186	11	8.6	0.70	96	0.70	8.49
		10	51	174	12	7.5	3.74	96	3.74	6.62
		10	51	177	12	7.9	3.54	96	3.54	6.42
		10	51	188	12	8.5	4.35	96	4.35	7.23
		10	51	184	12	8.6	5.34	96	5.34	8.22
	(3)	0.35	50	177	10	7.8	0.25	96	0.25	8.02
		1.2	50	177	10	7.9	0.46	96	0.46	5.88
		5.1	50	177	10	8.0	2.36	96	2.36	6.65
		10.4	50	177	10	7.9	3.54	96	3.54	6.31
		20.2	50	177	10	7.8	6.69	96	6.69	6.63
		40.9	50	177	10	7.7	12.20	96	12.20	6.18
		40.8	50	177	10	7.7	12.60	96	12.60	6.61
	(4)	<1.0	<4		10	7.0	3.7	96	3.7	9.4
		38	4		10	7.0	9.8	96	9.8	4.6
	(5)				10		1.6	24	0.8	
	(6)	1.4	8	25	10	6.2	0.5(0.9)	96	0.5(0.9)	5.9(6.3)

Table 2.3. — Continued.

Species	Source	Cl^{-1} (mgl^{-1})	Ca^{2+} (mgl^{-1})	Alkalinity ($CaCO_3$, mgl^{-1})	Temperature (^{o}C)	pH	LC50 NO_2-N	Time(hr) for LC50	LC50 adjusted to 96 hr	LC50 adjusted to 96 hr, 20 mgl^{-1} Cl^{-1}
		1.9	16	50	10	6.8	0.5(1.9)	96	0.5(1.9)	5.7(7.1)
		4.2	40	100	10	7.3	4.7(5.8)	96	4.7(5.8)	9.2(10.4)
		8.4	70	300	10	7.8	10.3(12.1)	96	10.3(12.1)	13.6(15.4)
		1.9	16	50	10	6.0	0.3(1.4)	96	0.3(1.4)	3.6(4.7)
		1.9	16	50	10	7.0	1.59	96	1.5(2.3)	4.8(5.6)
		1.9	16	50	10	8.0	2.5(3.6)	96	2.5(3.6)	5.8(7.1)
Chinook	(7)				15		0.88	96	0.88	
salmon	(8)	20		20	13	7.2	9.2	24	4.7	4.7
	(9)		32		9		5.75	48	4.6	
Cutthroat trout	(10)	0.44	53	176	12	8.0	0.52	96	0.52	6.6
Ictaluridae										
Channel	(11)			65	23	7.5	7.6	96	7.6	
catfish	(12)			220	22	8.7	12.8	96	12.8	
	(13)	22	80	190	32	7.9	7.1	96	7.1	6.4
Black bullhead	(14)	10	80				>40	>48	>32	>52
Cyprinidae										
Fathead	(3)	0.35	53	177	13	8.0	2.99	96	2.99	42
minnow		0.35	53	177	13	8.0	2.30	96	2.30	41

Table 2.3. — Continued.

Species	Source	Cl^{-1} (mgl^{-1})	Ca^{2+} (mgl^{-1})	Alkalinity ($CaCO_3$, mgl^{-1})	Temperature (^{o}C)	pH	LC50 NO_2-N	Time (hr) for LC50	LC50 adjusted to 96 hr	LC50 adjusted to 96 hr, 20 mgl^{-1} Cl^{-1}
	(15)	22	80	190	23	7.9	70	96	70	66
		22	80	190	23	7.9	45	96	45	41
European minnow	(16)				20		28	96	28	
Creek chub	(17)	9	27	98	18	8.3	81	24	>41	>63
Common carp	(14)	10					>40	28	>32	>52
Catostomidae										
White sucker	(14)	10					>100	48	>80	>100
Quillback	(14)	10					>100	24	>80	>100
Centrarchidae										
Largemouth bass	(15)	22	80	190	23	7.9	140	96	140	140
Bluegill	(18)	60			30	4.0	4.4	24	2.4	
		60			30	7.2	211.3	24	108	108
		5			30	4.0	4.6	24	2.4	
		5			30	7.2	282.0	24	144	144
Other families										
Mosquitofish	(19)			<100	22	7.3	1.5	96	1.5	
Blue tilapia	(15)	22	80	190	23	7.9	16.0	96	16.0	15
Logperch	(14)	10					<5	24	<3	<9

Table 2.3. -- Continued.

Species	Source	Cl^{-1} (mgl^{-1})	Ca^{2+} (mgl^{-1})	Alkalinity ($CaCO_3$, mgl^{-1})	Temperature (^{o}C)	pH	LC50 NO_2-N	Time (hr) for LC50	LC50 adjusted to 96 hr	LC50 adjusted to 96 hr, 20 mgl^{-1} Cl^{-1}
Brook stickleback	(14)	10					<5	24	<3	<9
Mottled sculpin	(3)	0.35	53	177	13	8.1	>67	154	>67	>106
Milkfish	(20)	26.8	49		27	8.0	12	48		
		8884.2	253		27	8.2	675	48		

(1) Russo et al. (1974); chloride from Russo and Thurston (1977)
(2) Russo et al. (1981); weighted means accorking to number of fish; pH treatments involve different anion treatments.
(3) Russo and Thurston (1977); calcium estimated from Thurston et al. (1978).
(4) Eddy et al. (1983).
(5) Smith and Williams (1974).
(6) Wedemeyer and Yasutake (1978); 5-g fish (10-g fish).
(7) Westin (1974).
(8) Perrone and Meade (1977); based on observed 50 % mortality, not a true LC50 analysis.
(9) Crawford and Allen (1977).
(10) Thurston et al. (1978).
(11) Konikoff (1975).
(12) Colt and Tchobanoglous (1976).
(13) Palacheck and Tomasso (1984b); calcium estimated from hardness.
(14) McCoy (1972); chloride values estimated from personal communication with city of Madison, Wisconsin.
(15) Palacheck and Tomasso (1984a); higher LC50 is for fish of 0.30-0.83 g, lower for fish of 0.9-3.33g; calcium estimated from hardness.
(16) Klingler (1957).
(17) Gilette et al. (1952).
(18) Huey et al. (1982); pH 4 probably unrealistic; 60 $mgl^{-1}Cl^{-}$ calculated from Cl^{-} : NO_2^{-} ratio for 24 hr LC50.
(19) Wallen et al. (1957).
(20) Almendras (1987).

Adapted from Lewis and Morris (1986).

2.7.2. ENVIRONMENTAL FACTORS

2.7.2.1. CHLORIDE

Prior to 1977, the literature on nitrite toxicity suggested great random variation in the toxicity of nitrite to fish, even within the same species. In 1977, however, Crawford and Allen showed that the toxicity of nitrite to small chinook salmon depended greatly on the salinity of the water in which the nitrite exposure occurred; mortality in sea water occurred at nitrite concentration 50 to 100 times higher than in fresh water. In the same year, Perrone and Meade showed that chloride could protect coho salmon against nitrite toxicity and proposed that chloride competes with nitrite for transport across the gills (also see Russo and Thurston, 1977). The effect of chloride on the toxicity of nitrite is now known to be so great that experiments in which chloride concentrations are not documented are of very little value because they cannot be meaningfully compared with the results of other studies.

Although several studies offer comparisons of toxicity at two or three chloride levels (Crawford and Allen, 1977; Wedemeyer and Yasutake, 1978; Almendras, 1987), very few are based on a sufficiently large number of chloride concentrations that the complete form of the relationship between chloride and toxicity can be discerned. One of the best studies on salmonids is that of Russo and Thurston (1977), who gave the results of nitrite toxicity tests at six

chloride concentrations for rainbow trout. Nitrite toxicity decreases with increasing chloride ion concentration, and the relationship between them is linear. The slope of the regression line of the relationship between 96 hr LC50 (Y, mgl^{-1} NO_2-N) and chloride concentration (X, mgl^{-1}) is 0.29: an increase of 1 mgl^{-1} in the concentration of chloride raises the 96 hr LC50 by 0.29 mgl^{-1} NO_2-N.

Tomasso et al. (1979) compared methemoglobin levels of channel catfish over a range of chloride and nitrite concentrations. It is clear from the experiments that chloride concentration is very important in governing methemoglobin formation. For example, channel catfish in water containing 3.54 mgl^{-1} chloride developed a 77 % methemoglobin level in the presence of 1.5 mgl^{-1} NO_2-N. In contrast, in the presence of 60 mgl^{-1} chloride and the same amount of nitrite, the methemoglobin level was indistinguishable from background (8.8 %).

Schwedler and Tucker (1983) did a study similar to that of Toamsso et al. (1979). Combining their own data with those of Tomasso et al., they concluded that the relationship between percent methemoglobin (Y) and their molar ratio of nitrite to chloride (X) was $Y = 7.33 + 78.17\ X$. Bowser et al. (1983) found that the 96 hr LC50 for channel catfish corresponded approximately to 80 % (70 - 90 %) methemoglobin, for which the molar ratio X is 0.94 according to the equation. The implication of this relationship is that the addition of 1 mM of chloride (35 mgl^{-1}) raises the 96 hr LC50 for nitrite by approximately 0.94 mM (13 mgl^{-1}).

Another relationship between chloride and nitrite can be derived from the combined data of Palachek and Tomasso (1984a), Russo and Thurston (1977), and McConnell (1985) for the fathead minnow. As shown by McConnell (1985) from regression analysis of this combined data set, 1 mM of chloride (35 mgl^{-1}) raises the 96 hr LC50 for nitrite by 5.08 mM (70 mgl^{-1}). Among the three species for which a chloride-toxicity relationship is available, rainbow trout and channel catfish are the most sensitive and the fathead minnow is considerably less sensitive. The effect of chloride on LC50 for nitrite appears to be inversely related to sensitivity of a fish species to nitrite: the most sensitive species benefit least from chloride addition, although the benefit is large even for sensitive fish. Possibly the less sensitive fish are able to discriminate to some degree against nitrite, and thus chloride is more potent in offsetting nitrite toxicity in these fish.

2.7.2.2. OTHER ANIONS

There is some information on the effects of anions other than chloride on nitrite toxicity. Bromide, which is similar chemically to chloride, was studied by Eddy *et al.* (1983), who found that 80 mgl^{-1} bromide was enough to offset completely the presence of 32 mgl^{-1} NO_2-N for Atlantic salmon in fresh water. Although this shows that chloride is not the only ion that can offset nitrite toxicity, bromide is not normally a major constituent of fresh waters.

Bicarbonate, although chemically very different from chloride, is of interest because it accounts for a high fraction of the total anions in fresh waters. The studies by Eddy et al. (1983) on Atlantic salmon included a test of the effect of sodium bicarbonate on nitrite toxicity. A concentration of 152 mgl^{-1} HCO_3^- did reduce toxicity significantly, but not nearly so much as bromide at 80 mgl^{-1}.

Huey et al. (1980) showed for channel catfish that 610 mgl^{-1} HCO_3^- was sufficient to hold methemoglobin levels at 8 %, the background level, whereas controls without bicarbonate showed 60 % methemoglobin at the experimental exposure of 0.76 mgl^{-1} NO_2-N. Given the general relationship already described for channel catfish, it is estimated that 2 mgl^{-1} (0.057 mM) of chloride, which is the amount required to offset 0.76 mgl^{-1} (0.054 mM) of NO_2-N, would have been required to hold methemoglobin levels near background. In contrast, 610 mgl^{-1} (10 mM) was required to achieve almost complete suppression of methemoglobinemia. Thus on a molar basis, the bicarbonate ion in this case was approximately 0.54 % as effective as the chloride ion.

Bath and Eddy (1980) showed that rainbow trout experiencing 90 % mortality at 9.8 mgl^{-1} NO_2-N (0.7 mM) after 72 hr showed 50 % survival under the same conditions in the presence of 152 mgl^{-1} (2.5 mM) bicarbonate. This experiment, like the one already mentioned, suggests that bicarbonate is effective, but not so effective as

chloride, in offsetting nitrite toxicity. Bowser et al. (1983) also found that bicarbonate was less effective than chloride in preventing methemoglobinemia. It has been reported that freshwater fish give out bicarbonate in exchange for chloride by the anion exchange mechanism. Thus chloride uptake from the medium is inhibited by bicarbonate and bicarbonate may repress nitrite uptake by a similar mechanism.

Russo et al. (1981) documented a small nitrate repression of nitrite toxicity. Divalent and trivalent anions that have been studied in connection with nitrite toxicity include sulfate, phosphate and borate. Huey et al. (1980) found that 1.42 gl^{-1} of sodium sulfate failed to reduce the methemoglobin percentages of channel catfish exposed to 0.76 mgl^{-1} NO_2-N. Eddy et al. (1983) showed that magnesium sulfate and potassium sulfate at sea water concentrations had very little effect on the toxicity of nitrite to rainbow trout. Russo et al. (1981) showed minimal effects of sulfate and phosphate on the toxicity of nitrite to rainbow trout.

In summary, the literature presently shows that chloride and bromide are highly effective in reducing nitrite toxicity, that bicarbonate and nitrate have detectable effects but are not nearly so effective as chloride and bromide, and that divalent and trivalent anions have very little effect on nitrite toxicity.

2.7.2.3. CATIONS

Calcium, magnesium, sodium, and potassium are typically present in considerable quantities in fresh- or sea- waters. The effects that these ions might have on nitrite toxicity are therefore of interest, however, most of the available information on this subject are concerned with calcium ion.

Crawford and Allen (1977) studied the effect of calcium and sea water on nitrite toxicity to chinook salmon. They found that the addition of calcium sulfate to fresh water and calcium-free artificial sea water decreased the toxicity of nitrite. These observations suggested that calcium is an important antagonist to nitrite toxicity and may be responsible in part for the very high nitrite tolerance seen in natural sea water experiments. However, the authors suggested that another factor was also involved because they found that raising the calcium concentration of the fresh water to the level of natural sea water decreased the toxicity of nitrite but did not reduce methemoglobinemia. Conversely, nitrite in calcium-free artificial sea water was highly toxic but did not induce appreciable methemoglobinemia. The dissociation of methemoglobinemia from mortality in the presence of calcium in these tests remains unexplained and has not yet been reported in other studies.

Although Bath and Eddy (1980) gave additional evidence for a possible connection between calcium and nitrite toxicity, they did not

separate the effect of calcium from that of anions (NO_3^-) added with calcium. For this reason, the results are inconclusive with regard to calcium.

Bowser et al. (1983) found that sodium chloride and calcium chloride provided equivalent protection against nitrite toxicity for channel catfish, suggesting that the identity of the metal cation was of little importance. Tomasso et al. (1980) provided similar information for channel catfish. Wedeneyer and Yasutake (1978) found nitrite to be markedly less toxic in the presence of calcium than of sodium, but their results are difficult to interpret because the LC50 values are very different from the others reported in their paper or elsewhere in the literature for similar chloride concentrations. Calcium has been reported to be able to reduce diffusion by altering membrane permeability (Potts and Fleming, 1970). Krous et al. (1982) also pointed out that high concentrations of calcium generally reduce the efflux of chloride through the gills. Perrone and Meade (1977) proposed that nitrite is actively pumped across the gills at the chloride uptake sites (chloride cells). If this is the case, the reduced branchial efflux of chloride, in turn, reduces the requirement for uptake of chloride, which decreases the uptake of nitrite. Conversely, reduced calcium concentration will stimulate chloride cell proliferation and activity in response to ion efflux, resulting in a higher uptake rate for chloride and available nitrite. Thus there are theoretical reasons to expect that calcium ions will reduce the toxicity of nitrite, although experimental work that has been done

thus far suggests that the effect is a weak one.

2.7.2.4. pH

The aqueous nitrite equilibrium NO_2^- + H^+ ⟷ HNO_2 is pH-dependent, however the effect of pH on toxicity of nitrite is still very uncertain. Contributions to the literature on this subject frequently are not definitive because they fail to separate the possible effects of anions from those of acidity or they use pH ranges outside the normal adaptive range of the fish.

Bath and Eddy (1980) reported for rainbow trout that acidity of the water had no significant effect on nitrite toxicity except at extreme pH levels (below pH 5 and above pH 10), but they did not give details of their findings. Working with coho salmon at two different chloride levels, Meade and Perrone (1980) showed that pH, which they controlled by means of a tris buffer, affected the plasma nitrite concentrations and plasma methemoglobin. Within a pH range of 6.5 to 8.0, they found that both methemoglobin and plasma nitrite were about twice as high at pH 6.5 as at pH 8.0 (15 mgl^{-1} Cl^- and 10 mgl^{-1} NO_2-N). The use of a buffer for manipulation of pH in such an experiment, however, changes the anion background. Thus it is difficult to separate the effects of changing anion background from the effects of acidity.

For channel catfish, Huey et al. (1980) adjusted the holding

water to a pH of 5.3 using a phosphate buffer and to a pH of 9.1 using a sodium bicarbonate buffer. Channel catfish held at 0.76 mgl^{-1} NO_2-N for 24 hour showed methemoglobin levels of 11 % at pH 9.1 and 76 % at pH 5.3. Because experimental evidence suggests that bicarbonate reduces the uptake of nitrite, the addition of 1,220 mgl^{-1} (20 mM) bicarbonate, as in these experiments, might well result in reduced methemoglobin levels even in the absence of a pH change. Thus it is difficult to interpret the experiments from the viewpoint of pH alone.

In other experiments, Huey et al. (1982) used pH 4 as the lower end of a pH test range for bluegill. Although there was substantial interaction between pH and nitrite toxicity because of a lower LC50 at pH 4, the results are probably not very meaningful because pH 4 is well outside the normal adaptive range of fish.

The strongest case for an pH effect is based on the work of Wedemeyer and Yasutake (1978) with steelhead. In these experiments, the pH of soft water was adjusted to 6, 7, and 8 with a weak phosphate buffer. If results for pH 6, which is extreme for steelhead in view of the unlikelihood that such a pH would occur in its natural environment, are discounted, the results showed 96 hr LC50s of 1.5 mgl^{-1} NO_2-N at pH 7 and 2.5 mgl^{-1} NO_2-N at pH 8 for fish weighing 5 g. The LC50s were 2.3 mgl^{-1} at pH 7 and 3.6 mgl^{-1} NO_2-N at pH 8 for fish weighing 10 g. The pH effect, although statistically significant, was small. Furthermore, it should be noted that pH adjustment of soft water changes the bicarbonate content of the water by influencing the

atmospheric equilibrium of carbon dioxide with water.

Experiments by Russo et al. (1981) on rainbow trout demonstrated that over a pH range from 6.44 to 8.10, there was no pattern in the 96 hr LC50 (correlation analysis; $P>0.05$). Above pH 8.1, the authors added sodium hydroxide to maintain high pH, and the 96 hr LC50 began to show a pH response. However, a pH in excess of 8.1 might be outside the adaptive range in view of its rarity in natural trout habitats; the experiments do not demonstrate a pH effect within the most environmental pH range.

In summary, in spite of frequent references to the role of pH in governing nitrite toxicity, there is no unequivocal evidence as yet for an effect of pH on nitrite toxicity over the normal environmental pH range of freshwater or seawater fishes. The interest of investigators in the importance of pH has been very high since the suggestion, based on experiments by Colt and Tchobanoglous (1976), that the amount of blood plasma nitrite of fishes is governed by the entry of undissociated nitrous acid through the gills. For example, Wedemeyer and Yasutake (1978) concluded that pH must have an important effect because it controls the balance between nitrite and nitrous acid, both in the medium and inside the fish. However, the proposition that nitrous acid is the main mode of entry of nitrite into fish is still very much open to question. As mentioned before, the weight of evidence at present favors the interpretion that the nitrite ion is pumped into the fish through the gills except in those

fish that are able to discriminate against nitrite (eg. largemouth bass), and that the role of pH in governing the entry of undissociated nitrous acid is unimportant. This does not rule out some roles for the hydrogen ion, however. For example, as pointed out by Meade and Perrone (1980), a change in the pH could alter the uptake characteristics of the ion-concentrating mechanisms in the gills, leading to pH effects over very large pH ranges. Over the most likely natural pH ranges, once the effects of different ionic backgrounds have been taken into account, the effect of pH on nitrite toxicity appears to be small.

2.7.2.5. TEMPERATURE

Temperature has multiple effects on aquatic ectotherms such as fish. Not only can temperature directly cause death (i.e. act as a lethal agent), nonlethal temperatures may influence the toxicity of chemicals, or alternately the ability of a fish to resist a particular chemical. Elevated temperature increases the diffusion of molecules through water, gill permeability and metabolic rates. The latter increases oxygen demand and hence gill lamellar blood flow. Increases in the rate of these internal and external process enhance chemical uptake, particularly in chemicals such as nitrite whose primary port-of-entry is the gill surfaces. Also Bath and Eddy (1980) suggested that fish can transport nitrite against a concentration gradient via a brachial anion exchange mechanism. Thus it is expected that the

toxicity of nitrite increases with increasing temperature.

The only experimental study of the relationship between temperature and nitrite toxicity appears to be for channel catfish (Colt and Tchobanoglous, 1976; Huey et al., 1984) Over a relatively small range (22 - 30° C), Colt and Tchobanoglous (1976) showed no significant relationship between nitrite toxicity and temperature. In the study of Huey et al. (1984), channel catfish held at 30°C in the presence of 0.91 mgl^{-1} NO_2-N over a period of 24 hours developed methemoglobin concentrations almost twice as high as those of fish held at 10°C. The higher methemoglobin level, however, did not necessarily indicate that greater lethality would occur at 30°C. Huey et al. (1984) found that the fish held at 30°C showed a more rapid return to background methemoglobin levels following a 12 hour recovery period in nitrite-free water. These data suggested that increases in internal responses such as biochemical transformation and depuration may serve to compensate for increase uptake rates at higher temperature. The activity of the methemoglobin-reductase system is optimized and result in greater loss of methemoglobin in the 30°C acclimation group.

The higher amount of oxygen in water and the lower metabolic rates of fish at lower temperatures might render nitrite a less potent toxin at lower temperatures. However, given that lower temperatures also reduce the efficiency of detoxification mechanisms, general

conclusions should be approached with caution.

Temperature-toxicity relationships are complex. This complexity exists because temperature can affect any or all of the separate environmental, biochemical, physiological processes such as chemical availability, uptake, internal processes may have its own unique relationship with temperature. Since fish species appear to possess different temperature optima, it is possible that temperature-toxicity relationship may need to be studied on a chemical-by-chemical and species-by-species bases.

2.7.2.6. OXYGEN

Oxygen can affect the toxicity of nitrite because nitrite reduces the oxygen-carrying capacity of the blood; reduction of the oxygen supply in the external medium will exacerbate the oxygen supply problem within the fish. In the only published study that bears on this qestion, Bowser et al. (1983) showed that an oxygen concentration of 5 mgl^{-1}, in the presence of nitrite, was not sufficient for channel catfish, even though channel catfish normally tolerate oxygen concentrations below this.

2.8. TREATMENT AND PREVENTION

Nitrite intoxication occasionally occurs and treatment will be necessary. A number of compounds have been used to treat or prevent methemoglobinemia, of them methylene blue and ascorbic acid are most commonly used. Commercial fish farmers usually administer high dietary ascorbic acid for the cultured fish to prevent methemoglobinemia. Wedmeyer and Yasutake (1978) have demonstrated that methylene blue at 0.1 or 1.0 mgl^{-1} was effective in decreasing acute nitrite toxicity. For alleviating methemoglobinemia, removing the fish to fresh water was about as effective as 0.1 mgl^{-1} treatments. However, 1 mgl^{-1} methylene blue did dramatically reduce the methemoglobinemia during the first few hours which would probably be a critical advantage in treating severely poisoned fish.

The rapid prevention of nitrite - induced methemoglobinemia and mortality provided by chloride and bicarbonate ions indicate that nitrite crises encountered would include replacing contaminated water with fresh water or the addition of monovalent anions. If these methods can be administered with little disturbance of severely stressed fish, recovery from methemoglobin should occur within 24 hours (Huey et al., 1980).

CHAPTER THREE DETERMINATION OF THE 96 HOUR MEDIAN LETHAL CONCENTRATIONS OF NITRITE FOR *LATES CALCARIFER* IN FRESH WATER, 15 $^{o}/_{oo}$ SEA WATER AND 32 $^{o}/_{oo}$ SEA WATER

3.1. SUMMARY

1. The 96 hour median lethal concentrations of nitrite for Lates calcarifer fingerlings in fresh water, 15 $^{o}/_{oo}$ sea water and 32 $^{o}/_{oo}$ sea water were determined by static bioassay at 27 ^{o}C.

2. The 96 hour median lethal concentrations of nitrite were estimated to be 14.5 mgl^{-1} NO_2-N in fresh water, 104 mgl^{-1} NO_2-N in 15 $^{o}/_{oo}$ sea water and 93 mgl^{-1} NO_2-N in 32 $^{o}/_{oo}$ sea water.

3. The tolerance of L. calcarifer to nitrite toxicity was compared with other fish species on which there are published data.

3.2. INTRODUCTION

In measuring the toxicity of a chemical, the objective is to estimate the range of chemical concentrations that produce a deleterious effect on groups of test organisms under controlled conditions. The results of the exposure are plotted on a graph that relates the concentration of the test chemical to the percentage of organisms in test groups exhibiting the defined response. Such a correlation is commonly referred to as a concentration-response relationship. Generally, within certain limits, the greater the concentration of the test chemical, the more severe the response. The curve drawn to represent this relationship will generally be asymptotic (Fig. 3.1) since at all concentrations below some minimum (threshold) value no measurable adverse response will be elicited, while at all concentrations above some maximum value most or all of the test groups will be adversely affected. The concentration at which 50 % of the individuals react (the median) after a specified length of exposure (eg. 24 or 48 hours) is therefore used as a measure of the toxicity of the chemical.

Toxicity tests with aquatic organisms can be conducted by administering the material directly by injection or incorporation into food. However, for materials that are soluble in water, tests are conducted by exposing groups of organisms to several treatments in which different concentrations of the material are mixed in water.

Death is an easily detected deleterious response, thus for the initial test measurement in toxicological evaluation, it is customary to use mortality as an index. Other parameters measured include growth rate, reproduction, pathology, biochemistry, physiology and behaviour. The concentration estimated to produce mortality in 50 % of a test population over a specific time period is termed median lethal concentration (LC50) (Parrish, 1985). The length of exposure is usually 24 to 96 hours,depending on the species. The 96 hour duration of the acute toxicity test is used as a standard because it generally covers period of acute lethal action. Therefore, the measure of acute toxicity most frequently used with fish and macroinvertebrates is the 96 hour median lethal concentration (96 hr LC50). However, some animals such as daphnids and oyster larvae are exposed for only 48 hours (or less) because of problems associated with longer exposure times such as starvation (Parrish, 1985).

Fig. 3.1.a is an example of a mortality curve in which the mean percent mortality for each test group has been plotted on an arithmetic scale against the concentration producing that mortality. The response of test organisms to the different chemical concentrations yields a characteristic S-shaped curve. The LC50 can be interpolated from the curve by drawing a horizonal line from the 50 % mortality point on the ordinate to the curve and then drawing a vertical line from the point of intersection with the curve to the abscissa. The normally distributed sigmoid curve approaches a

mortality of 0 % as the concentration is decreased and approaches 100 % as the concentration is increased. The middle portion of the curve, in the region between 16 and 84 %, is almost linear. These values represent the limits of 1 standard deviation (SD) of the mean (and the median) in a normally distributed population of organisms. In a normally distributed population, the mean $\pm$ 1 SD represents 68.3 % of the test population, the mean $\pm$ 2 SD 95.5 % of the test population, and the mean $\pm$ 3 SD 99.7 % of the test population (Rand and Petrocelli, 1985).

Fig. 3.1.b is a plot of the data in Fig. 3.1.a, but with the concentration shown on a logarithmic scale (transformed data). The sigmoid shape is again evident but the curve approaches a straight line. Fig. 3.1.c represents another transformation of the same data, with the logarithm of concentration plotted against percent mortality expressed as probits. The probit transformation adjusts mortality data to an assumed normal population distribution, which results in a straight line being the best fit curve. The LC50 is obtained by drawing horizontal and vertical lines in the same manner described for Fig. 3.1.a.

The logarithmic conversion was introduced by Krogh and Hemmingsen (1928, cited in Rand and Petrocelli, 1985) and later by Gaddum (1933, cited in Rand and Petrocelli, 1985). Since concentration-response data are normally distributed, the percent response was converted to units of deviation from the mean or normal

equivalent deviations (NEDs) by Gaddum (1933, cited in Rand and Petrocelli, 1984). The NED for a 50 % response is zero and that for an 84.1 % response is +1. Bliss (1934 a,b, cited in Rand and Petrocelli, 1985) later suggested that 5 be added to the NED to eliminate negative numbers. The converted units of NED plus 5 were called probits. The LC50 estimated by graphical interpolation is accurate and usually similar to the LC50 obtained by formal statistical analysis (Rand and Petrocelli, 1985).

The toxicity of nitrite to aquatic animals has received considerable attention over the past ten years. The median lethal concentration of nitrite has been widely studied in many freshwater fish species, but in only few euryhaline or seawater fish species (Crawford and Allen, 1977).

The present study aims at determining the 96 hour median lethal concentrations of nitrite for the euryhaline fish, Lates calcarifer, in fresh water, 15 $^{o}/_{oo}$ sea water and 32 $^{o}/_{oo}$ sea water. L. calcarifer is an economically important food fish in Hong Kong. In recent years, intensive aquaculture of L. calcarifer has developed very rapidly due to the economic feasibility of commercial production of this food fish. The data derived from this study hopefully can contribute to a better and efficient management of this species if closed culture systems are employed.

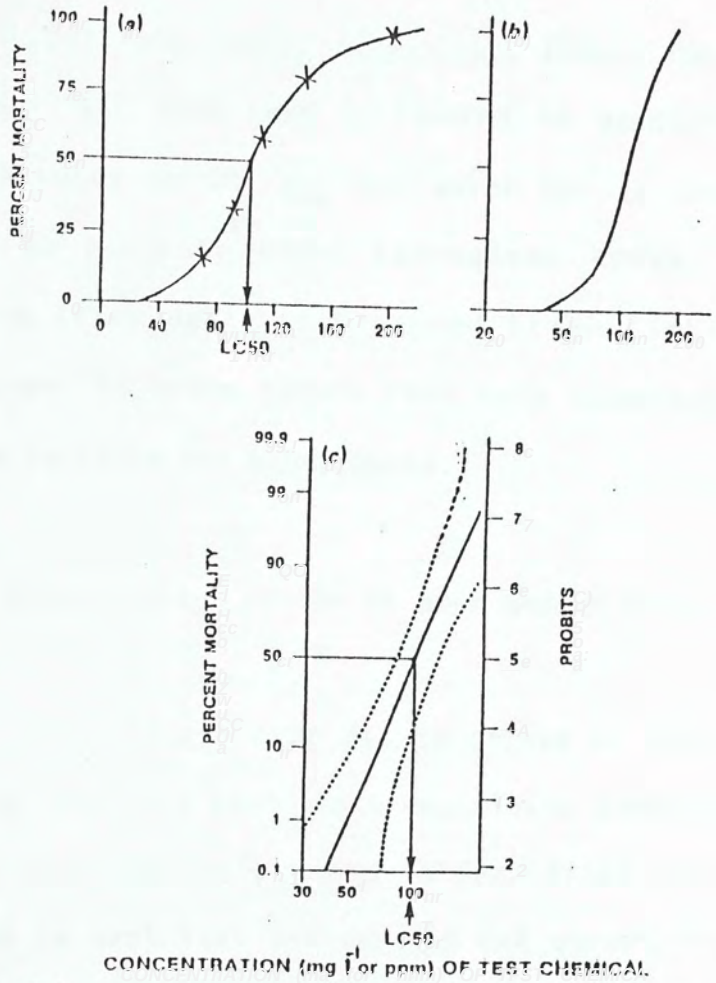

Figure 3.1. Mortality in a fish population exposed to a range of concentrations of a chemical in water. (*a*) Percent mortality versus concentration plotted on an arithmetic scale. (*b*) The same data as in (*a*), but with mortality on an arithmetic scale and concentration on a logarithmic scale. (*c*) The same data as in (*a*), but with mortality expressed as probits versus concentration on a logarithmic scale. The dotted lines on each side of the curve represent the 95% confidence limits.

Adapted from Rand and Petrocelli (1985).

3.3. Materials and Methods

3.3.1. Experimental animals

Sea bass, Lates calcarifer, fingerlings were purchased from Thailand. All fish were acclimated to dechlorinated tap water, 15 $^{o}/_{oo}$ sea water or 32 $^{o}/_{oo}$ sea water for at least 4 weeks prior to testing in separate 400-l fibreglass tanks. They were fed to satiation twice daily with minced trash fish meat. Feeding was discontinued 24 hours before fish were transferred into experimental tanks to initiate the experiments.

3.3.2. Determination of the 96 hour median lethal concentrations

Fish (8.2 $\pm$ 0.28 g), in groups of twelve, were transferred at random into 20-l test tanks containing dechlorinated tap water, 15 $^{o}/_{oo}$ sea water or 32 $^{o}/_{oo}$ sea water. After adapation for 24 hours, the water in each tank was renewed and except the control, each tank was dosed with an appropriate amount of reagent grade sodium nitrite to produce the required test concentrations. The test water was constantly aerated to maintain the dissolved oxygen content at saturation. The tests were conducted at 26-28 ^{o}C. The pH of 15 $^{o}/_{oo}$ and 32 $^{o}/_{oo}$ sea water was 8.0, and the pH of fresh water was adjusted to 8.0 by the addition of sodium hydroxide. Water characteristics and the nitrite concentrations were measured at the beginning and the end of the tests and were found to vary by less than 5 %.

Mortalities were monitored every 12 hours and totalled after 96 hours. The criteria for death are lack of gill movement and lack of reaction to gentle prodding. Dead individuals were removed from the tank, and death was confirmed by the inability to recover in nitrite-free water. The 96 hour median lethal concentration were determined according to the method of Litchfield and Wilcoxon (1949).

Test water nitrite concentrations were determined by the Azo-dye method as described by Strickland and Parsons (1972). The pH was measured with a research grade pH meter and the salinity with a refractometer.

3.4. Results

The mortality curves of Lates calcarifer upon exposure to nitrite in fresh water, 15 $^{o}/_{oo}$ sea water and 32 $^{o}/_{oo}$ sea water were presented in Fig. 3.2, Fig. 3.3 and Fig. 3.4. In order to estimate the 96 hour median lethal concentrations more accurately, the same data were plotted on a probability graph with mortality expressed as probits versus nitrite concentration on a logarithmic scale. The 96 hr LC50s were estimated to be 14.5 mgl^{-1} NO_2-N in fresh water (Fig. 3.5), 104 mgl^{-1} NO_2-N in 15 $^{o}/_{oo}$ sea water (Fig. 3.6) and 93 mgl^{-1} NO_2-N in 32 $^{o}/_{oo}$ sea water (Fig. 3.7).

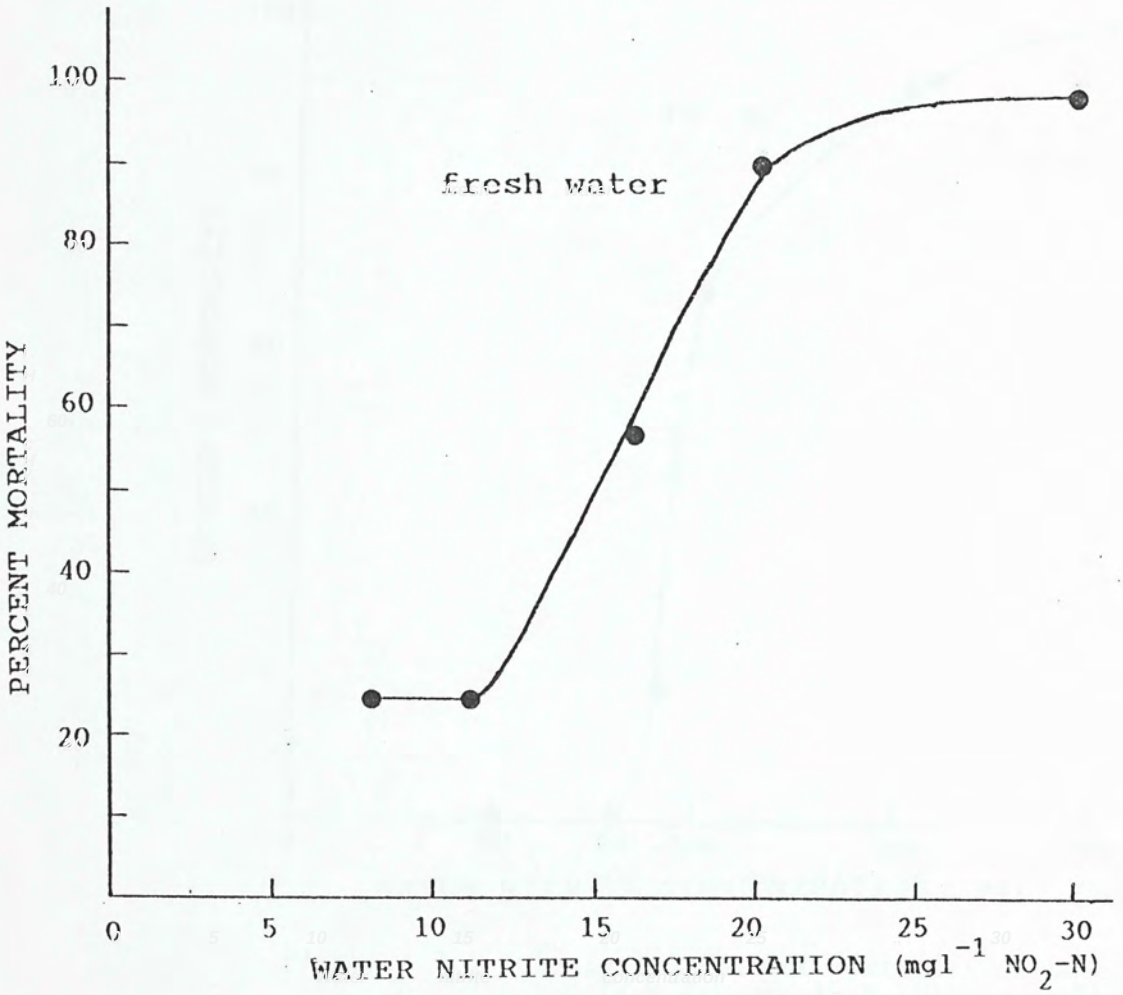

Figure 3.2. Culmulative percent mortality of Lates calcarifer exposed to a range of concentrations of nitrite for 96 hours in fresh water.

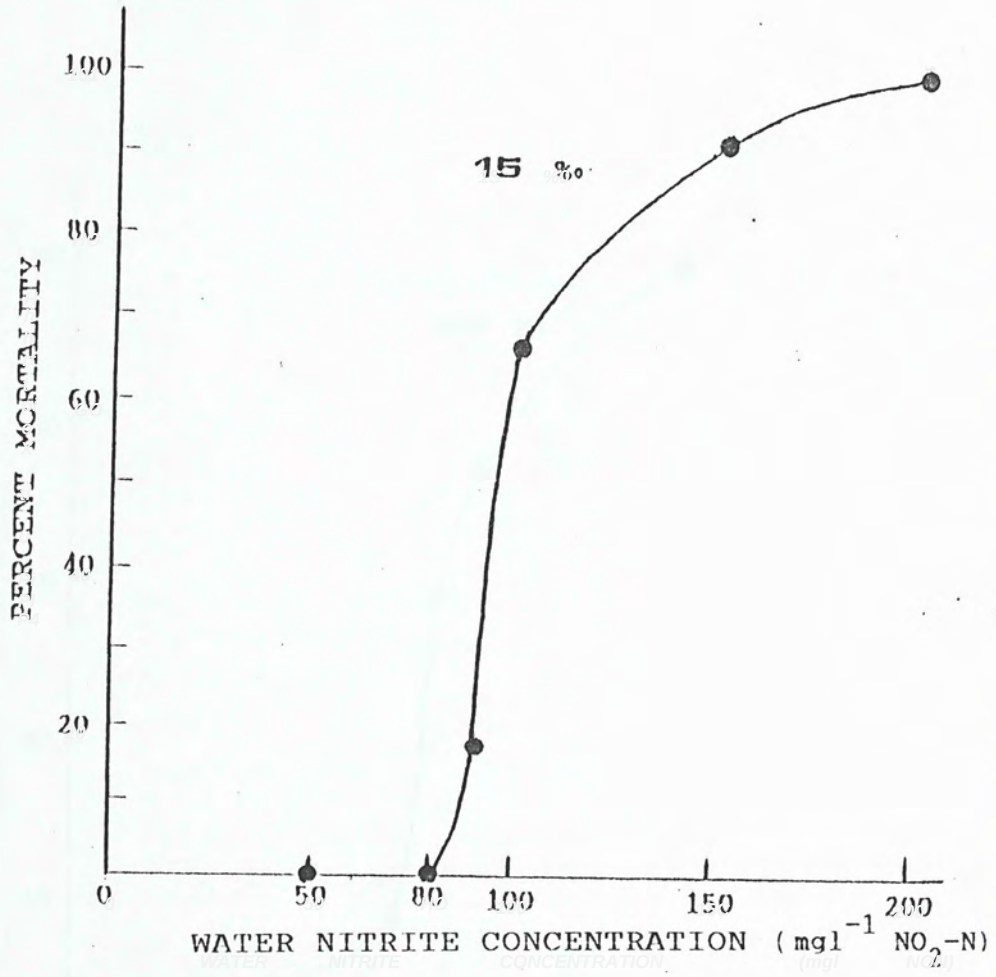

Figure 3.3. Culmulative percent mortality of Lates calcarifer exposed to a range of concentrations of nitrite for 96 hours in 15 ‰ sea water.

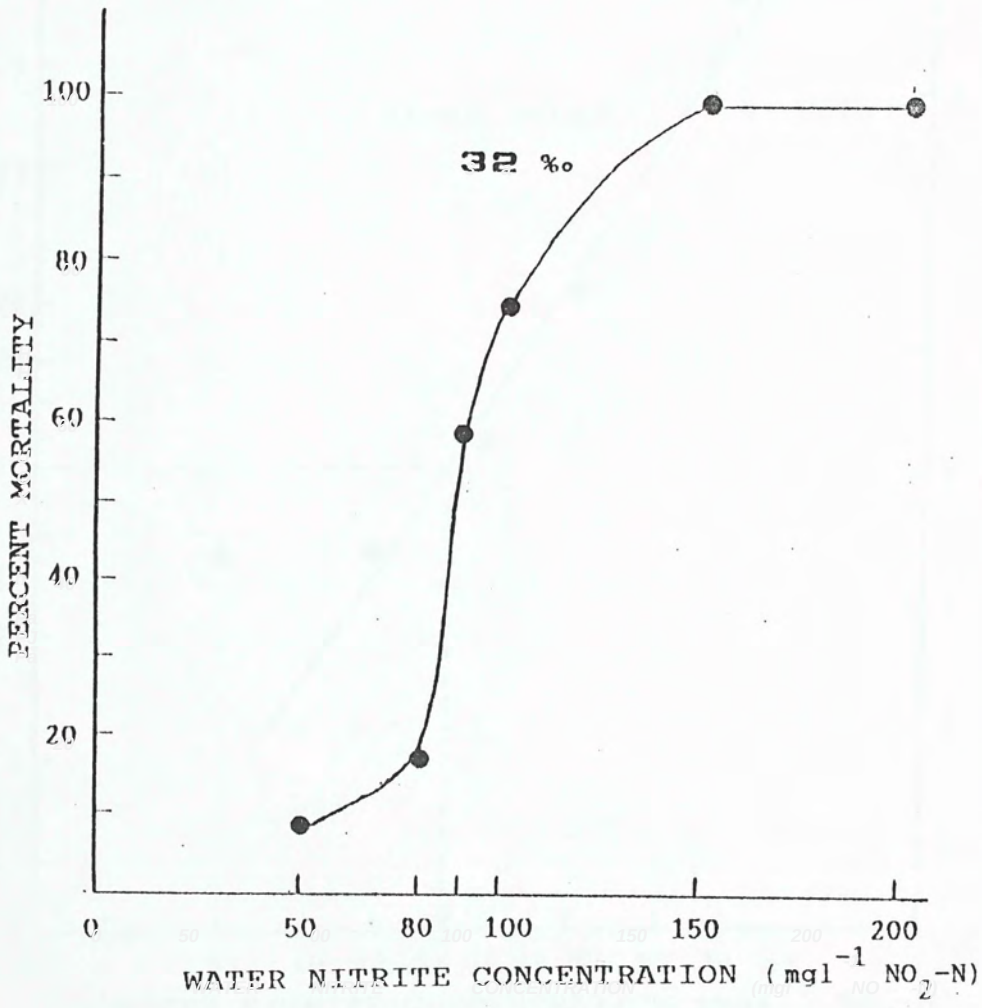

Figure 3.4. Culmulative percent mortality of Lates calcarifer exposed to a range of concentrations of nitrite for 96 hours in 32 ‰ sea water.

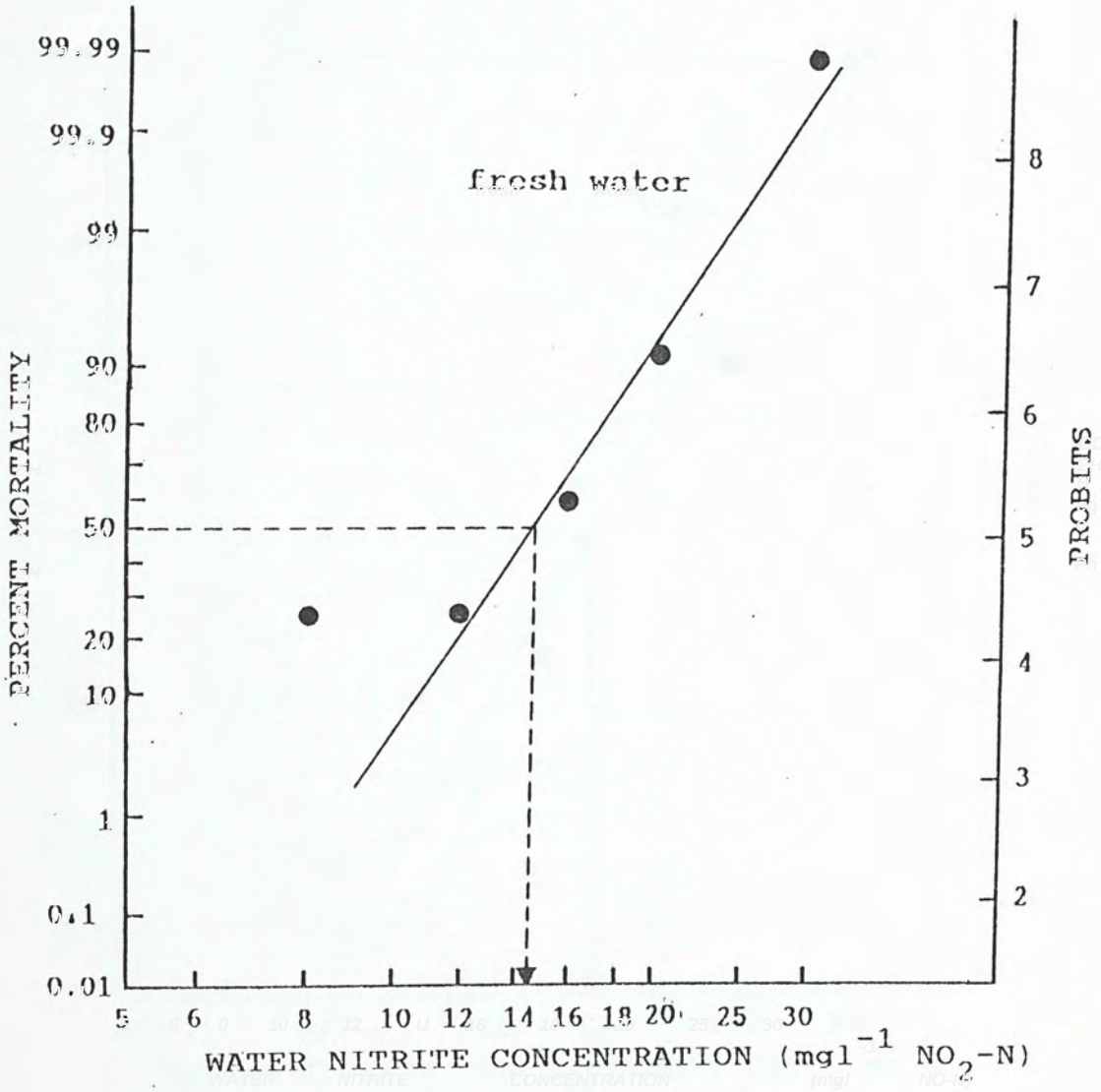

Figure 3.5. Estimation of 96 hour median lethal concentration of nitrite for Lates calcarifer in fresh water.

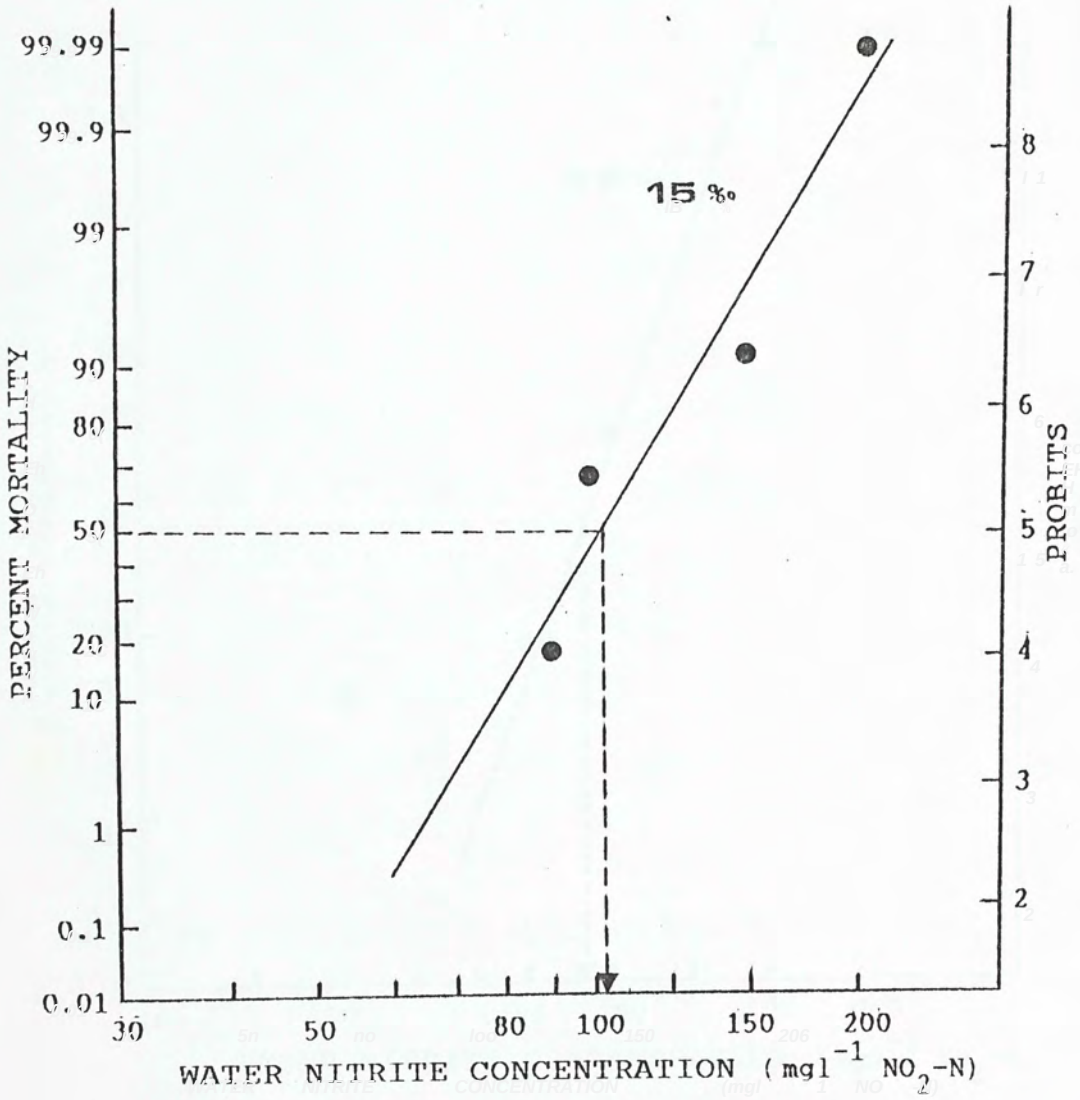

Figure 3.6. Estimation of 96 hour median lethal concentration of nitrite for Lates calcarifer in 15 ‰ sea water.

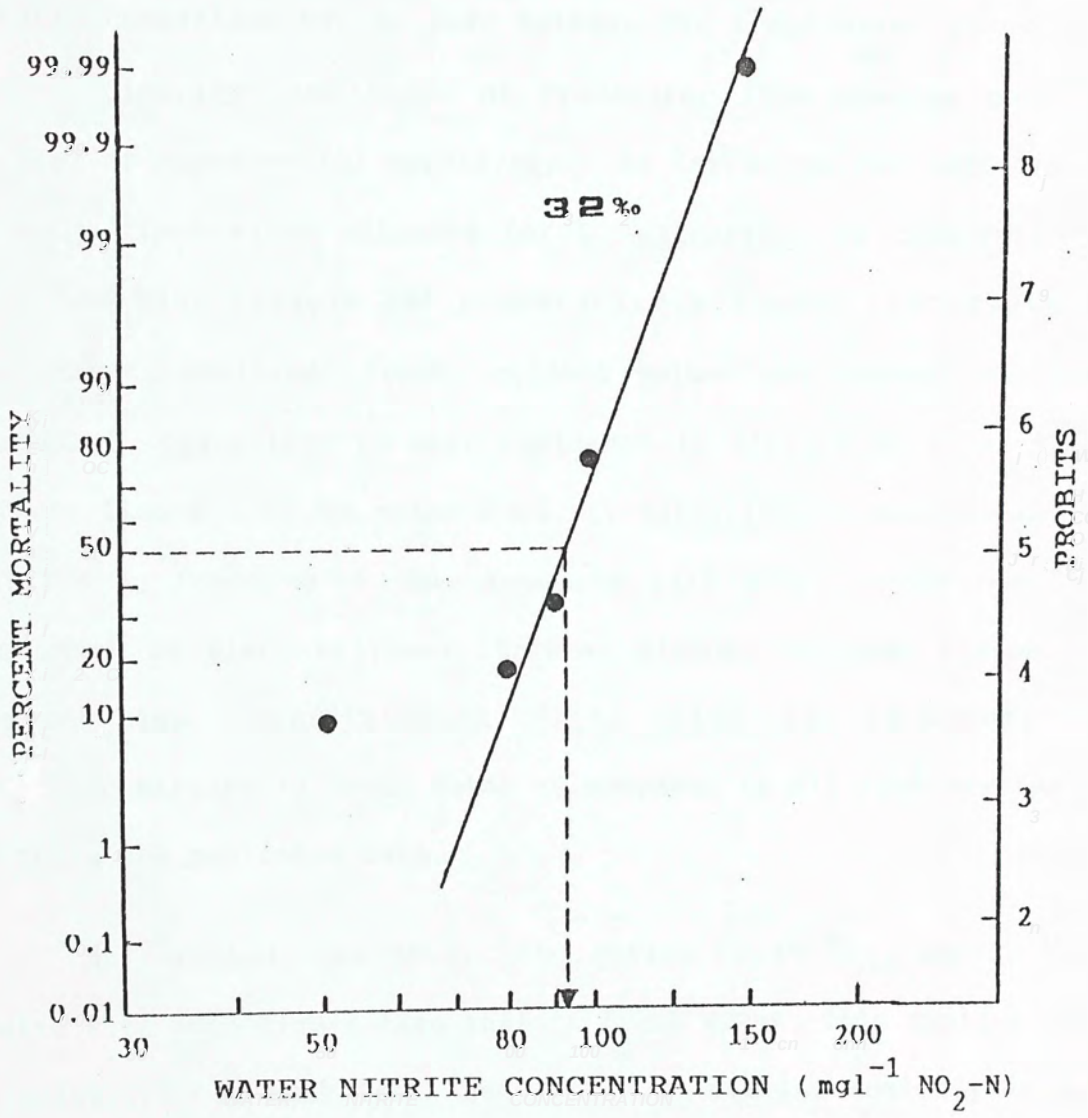

Figure 3.7. Estimation of 96 hour median lethal concentration of nitrite for Lates calcarifer in 32 ‰ sea water.

3.5. DISCUSSION

Due to the different experimental conditions, it is quite inappropiate to compare all the results obtained in the present study with those reviewed by Lewis and Morris (1986) (Table 2.3). However, reasonable comparison can be made between the fresh water 96 hr LC50 of Lates calcarifer and those of freshwater fish species obtained under similar experimental conditions. As far as we can see, the 96 hr LC50 in fresh water obtained for L. calcarifer is comparable to that of the blue tilapia but comparatively higher than those of rainbow trout, cutthroat trout, chinook salmon and channel catfish. That means L. calcarifer is more resistant to nitrite in fresh water than these fishes. On the other hand, L. calcarifer is more sensitive to nitrite in fresh water when compared with other freshwater fish species such as black bullhead, fathead minnow, European minnow and largemouth bass. Overall speaking, L. calcarifer is moderately resistant to nitrite in fresh water as compared to all fish species on which there are published data.

As expected, the 96 hr LC50 values in 15 $^{o}/_{oo}$ and 32 $^{o}/_{oo}$ sea water were much higher than that in fresh water. This implies that Lates calcarifer is much less susceptible to nitrite toxicity in sea water than in fresh water. The present study therefore provides additional support on the protective effects of ions such as chloride, bromide and calcium against nitrite toxicity as sea water contains a high quantity of these ions. The 96 hr LC50 value in 15 $^{o}/_{oo}$ sea

water was found to be higher than that in 32 $^{o}/_{oo}$ sea water. This may be due to the fact that 15 $^{o}/_{oo}$ sea water is roughly isotonic with the body fluid of the fish. It is possible that less energy is expended in osmoregulatory work and thus more energy can be channelled to resist the toxicity of nitrite. It is worth emphasizing that energy savings in an isotonic medium are considerable as the metabolic work of osmoregulation has been shown to be as high as 30 % of the total energy budget (Rao, 1968).

CHAPTER 4 EFFECTS OF NITRITE ON THE SHORT-TERM GROWTH OF *LATES CALCARIFER*

4.1. SUMMARY

1. The effects of nitrite on the growth of Lates calcarifer fingerlings in 32 $^{o}/_{oo}$ sea water were investigated.

2. The short-term growth rate (on a wet weight basis) of the fish was shown to be reduced at nitrite levels of 7 and 20 mgl^{-1} NO_2-N.

3. In spite of its great tolerance to high concentrations of nitrite as reflected in the high value of 96 hr LC50, comparative low nitrite levels resulted in growth suppression in the sea bass.

4. The growth response of the fish under nitrite intoxication was variable and this could largely be attributable to the great individual difference in susceptibility to nitrite toxicity.

4.2. INTRODUCTION

In the aquatic environment organisms are not usually exposed to high, acutely toxic concentrations of chemicals unless they are restricted to the vicinity of a chemical release site or spill area. Beyond the initial impact area dilution and dispersion occur and thus decreasing these acute concentrations to lower, sublethal levels. Therefore, organisms are generally exposed to sublethal concentrations of chemicals rather than to acutely toxic lethal concentrations. The lower concentrations may not produce death, but they may have a pronounced effect on the future survival of the organisms (Rand and Petrocelli, 1985).

In aquaculture, an important sublethal effect of a toxicant that warrants consideration will be its effect on growth. A toxicant may not cause mortality which definitely results in great economical loss, however, it may suppress the growth of cultured organisms resulting in low yield and similar economical loss.

A review of the literature showed that studies concerning the possible deleterious effects of nitrite on fish growth have only been done on steelhead and channel catfish. The growth of the juvenile channel catfish was found to be reduced at levels of 1.6 mgl^{-1} NO_2-N (one-fifth of the 96 hr LC50) and above (Colt *et al.*, 1981). However, no significant growth suppression was observed in steelhead at concentrations as high as 10 % of the 96 hr LC50

(Wedemeyer and Yasutake, 1978).

The work reported here was undertaken to provide information about the effects of nitrite on the short-term growth of Lates calcarifer fingerlings in 32 $^{o}/_{oo}$ seawater. To the best of the author's knowledge, the toxic effect of nitrite on the growth of L. calcarifer has not been hitherto reported.

4.3. MATERIALS AND METHODS

4.3.1. Experimental animals

Lates calcarifer fingerlings obtained from Thailand were kept in well-aerated 32 $^{o}/_{oo}$ sea water and were fed to satiation twice daily with minced trash fish meat. The fish were acclimated for at least 2 weeks prior to the start of the experiment.

4.3.2. Determination of growth rate

Eighteen fingerlings were randomly selected and individually distributed to the growth chambers which were 20-l fiberglass tanks containing 32 $^{o}/_{oo}$ sea water. The fingerlings were divided into two groups: group A and group B. The initial wet weight of group A and group B were 4.46 $\pm$ 0.11 g and 3.61 $\pm$ 0.12 g respectively. The fish were fed minced trash fish meat equivalent to 8 % of their body weight per day. The wet weight of the fish was measured once every 10 days. After a 40 day cultivation period, with group A serving as the control, group B was exposed to a nitrite concentration of 20 mgl^{-1} NO_2-N (one-fifth of the 96 hr LC50 in 32 $^{o}/_{oo}$ sea water). Similarly, the fish of the control and test groups were weighed once every 10 days and the mean % increases in wet weight of the two groups were compared.

During the whole experimental period, water temperature was

maintained at 25-28 ^{o}C. The dissolved oxygen was constantly kept at saturation by air pump. The pH varied from 7.8-8.1 and the salinity ranged from 31-33 $^{o}/_{oo}$.

4.4. RESULTS

The results derived from the growth experiment are presented in Fig. 4.1. For the first 40 days of the culture period, the growth rates of group A and B were very consistent and comparable. The mean % increase in wet weight was found to be 215.27 ± 12.60 % and 215.42 ± 21.16 % in group A and B respectively at Day 40. The growth rates of the two groups were not significantly different. After this 40 day culture period, with group A serving as the control, group B was immersed in 20 mgl^{-1} NO_2-N. In the first 3 days of nitrite exposure (Day 43), treated fingerlings did not show any abnormal behaviour except that they lost their appetite. In the following days, the response of the fish was quite variable. Some of them seemed to adapt to such a nitrite concentration and resumed their feeding, while others exhibited some abnormal behaviour including darkening of body colour, complete loss of appetite and irritant swimming. Using Day 0 as base line, the mean % increase in wet weight of group B at Day 50 was found to be 185.14 ± 19.88% which was significantly lower than the respective value (306.85 ± 16.88 %) of the control group. This indicates that during the 10 day nitrite exposure period (Day 40 - 50) there was actually a decrease of 30.28 ± 9.14 % in the wet weight of group B. As time passed, the condition of some treated fish deteriorated and 3 out of 9 fish in the treated group died between Day 52 and 54. Therefore, the comparison of the growth rate between group A and B had to be terminated at Day 50 of the growth period.

In order to investigate the impacts of a lower concentration of nitrite on growth, the growth rate of group A was monitored continuously for a period of 80 days and this was found to be rather consistent. Exposure to 7 mgl^{-1} NO_2-N was started at Day 81 and continued for 20 days. The response of the fish was very similiar to that encountered in the 20 mgl^{-1} NO_2-N exposure. Although this time all of the treated fish could survive and some of them even gained weight, the mean % increase in wet weight was reduced from 435.59 $\pm$ 31.89 % at Day 80 to 388.21 $\pm$ 39.39 % at Day 100. After removal of nitrite, the fish recovered very quickly and resumed their growth unexpectedly well, though at a slower rate.

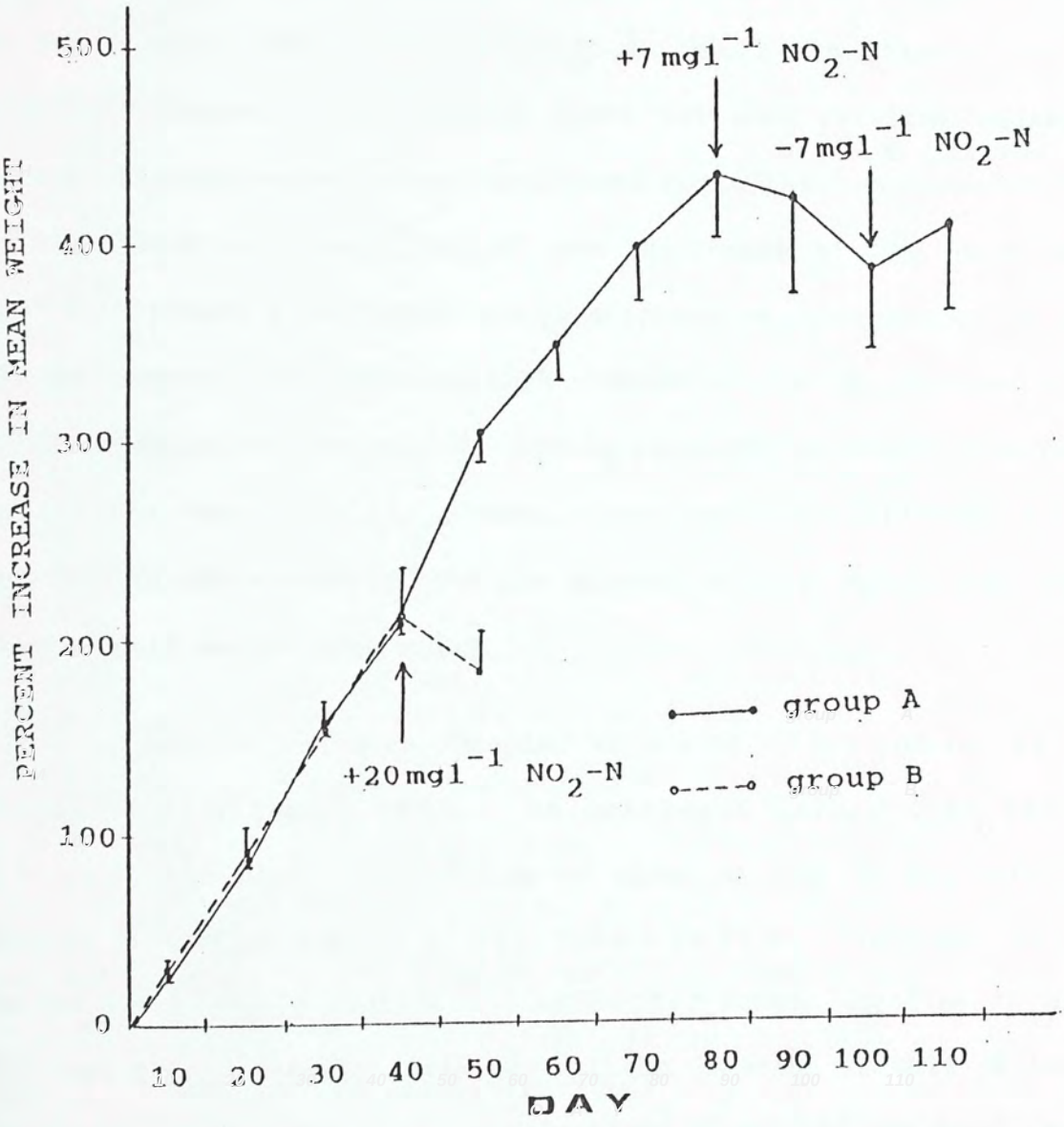

Figure 4.1. Effects of nitrite exposure on the short-term growth of Lates calcarifer (n = 9).

Values are presented as mean ± SEM

4.6. DISCUSSION

With reference to the high 96 hr LC50 value in 32 $^{o}/_{oo}$ sea water (93 mgl^{-1} NO_2-N), a comparative high sublethal concentration of 20 mgl^{-1} NO_2-N (one-fifth of the 96 hr LC50) was selected for the growth experiment. This nitrite level not only resulted in serious growth retardation but also significant mortality. A relative lower nitrite level of 7 mgl^{-1} NO_2-N (one-thirteenth of the 96 hr LC50) similarly caused significant growth suppression. Therefore, in spite of their great tolerance to high concentrations of nitrite in sea water, comparative low nitrite levels resulted in growth suppression in the sea bass. In the present study only two sublethal nitrite levels were administered, thus the minimal nitrite levels that reduce growth could not be determined.

Nitrite has been regarded as a slow-acting poision by some researchers (Klinger, 1957). As mentioned before (see Section 2.7.2.1.), the high concentration of chloride ions in sea water can provide protection against nitrite uptake in fish. Therefore, nitrite in sea water should require a considerably longer duration to exert its toxicity. In acute toxicity test the duration is only 96 hours, thus only sufficiently high concentrations of nitrite can exert lethal effects such as mortality on organisms. This can account for the high 96 hr LC50 value of nitrite in sea water. On the other hand, growth experiments usually extend over longer period of time, therefore a comparative low dosage of nitrite is enough to result in growth

suppression or even mortality.

As a result of microbial oxidation of ammonia, the main excretory product of fish, an intermediate product, nitrite is produced. Accumulation of nitrite is one of the most critical problems encountered in intensive aquaculture or in closed water reuse systems for fish culture. Since a low dosage of nitrite is sufficient to suppress fish growth and thus reducing fish yield, it is necessary to keep the nitrite concentration as low as possible in order to provide an optimal environment for fish growth and survival. For example, in rearing salmonid fish by intensive methods in a closed recirculating system, according to Westin (1974), a nitrite concentration of 0.012 mgl^{-1} must not be exceeded to obtain optimal growth of fish. Today in fish farming there exists a number of different systems for removing metabolic end products, especially nitrogenous compounds, and for reconditioning the water for reuse. Various types of biological filters (eg. trickling type, submerged type, updraft type, biodiscs, gravel bed, activated sludge type) have been designed and are mainly used today, in addition to chemical and physical methods of waste water treatment (Nagel, 1980).

CHAPTER FIVE CHANGES IN HEMATOLOGICAL PARAMETERS AND BLOOD RESPIRATORY PROPERTIES OF *LATES CALCARIFER* IN RESPONSE TO ACUTE AND PROLONGED NITRITE EXPOSURE

5.1. SUMMARY

1. Changes in hematological parameters and blood respiratory properties of *Lates calcarifer* in response to acute and prolonged nitrite exposure were studied.

2. When *L. calcarifer* was subject to prolonged (8 day) exposure of 10 mgl^{-1} NO_2-N, there were significant increases in blood nitrite concentration and methemoglobin content. On the contrary, a significant decrease in total hemoglobin was observed. An increase in methemoglobin content together with a reduction in total hemoglobin further decreased the functional hemoglobin content. There was no significant change in hematocrit and red blood cell count. The possible reasons for a lowered total hemoglobin and its impact on the fish were discussed.

3. Hematological parameters and venous blood oxygen tension of *L calcarifer* after acute (96 hour) exposure to 15, 20, 30, 50 and 80 mgl^{-1} NO_2-N were determined. Methemoglobin formation was highly correlated with the test nitrite concentrations. The methemoglobin content (as percent of total hemoglobin) of fish immersed in concentration of 96 hr LC50 was estimated to be 83 %. Venous blood oxygen tension tended to decrease as nitrite concentration increased. The decrease was probably due to a real decrease in oxygen content of the blood or an enhancement in metabolic requirement so that more oxygen would be depleted from the blood stream.

4. Blood oxygen dissociation curve showed that L. calcarifer has a relatively low oxygen affinity (P_{50} = 14.5 ± 2.28 mmHg) with Hill coefficient (n) of 1.46. Exposure of the fish to 50 mgl^{-1} NO_2-N resulted in a leftward and upward shift of blood oxygen dissociation curve with P_{50} = 5.93 ± 1.64 mmHg and n = 0.99. The increased blood oxygen affinity in treated fish can facilitate the oxygen loading from water to the blood stream at the respiratory surfaces.

5. Nitrite-treated fish had lowered arterial and venous blood oxygen tension, arterial and venous oxygen content, and blood oxygen capacity. However, no change in venous blood pH was observed between control and nitrite-treated fish.

6. The hematological data suggested that a hypoxic state was not evident in nitrite-treated Lates.

5.2. Introduction

It is well known that both hematological parameters and blood respiratory properties change when freshwater fish are exposed to nitrite (Margiocco et al., 1983; Wilmy et al., 1987). Typically, freshwater fish respond to nitrite exposure by methemoglobin formation (eg. Brown and McLeay, 1975; Huey et al., 1980; Wilmy et al., 1987). Other hematological responses reported include reductions in total hemoglobin (Brown and McLeay, 1975; Margiocco et al., 1983; Wilmy et al., 1987), hematocrit (Eddy et al., 1983; Wilmy et al., 1987) and erythrocyte count (Wilmy et al., 1987). In addition, the blood of nitrite-exposed freshwater fish has a higher oxygen affinity and a more pronounced Bohr effect (Bartlett et al., 1987). As methemoglobin is unable to bind with oxygen, methemoglobin formation can severely reduce the blood oxygen carrying capacity (Bodansky, 1951). Some authors (eg. Huey et al., 1980; Wilmy et al., 1987) have proposed that heavy nitrite loads producing high methemoglobin contents may cause death as a result of hypoxia or anoxia. It has been reported that fish respond to hypoxia by an increase in hematocrit, hemoglobin concentration and blood oxygen capacity (Wood et al., 1975; Smit and Wattingh, 1978). Blood oxygen affinity is also increased by a decrease of red cell NTP concentration (Wood, 1980). Therefore, the hematological responses triggered by nitrite exposure are unlikely due to hypoxia per se.

Despite the well documentation of the effects of nitrite in

freshwater fish, similar studies on marine fish have been limited to only one species (Dicentrarchus labrax). In this marine species, as nitrite exposure time or exposure concentration increased, total and functional hemoglobin concentrations decreased while percent methemoglobin increased (Scarano et al., 1984).

Since the hematological parameters and blood respiratory responses of euryhaline fish to nitrite exposure are not well studied compared with those of the freshwater fish, it is the objective of this study to compare the hematological parameters (total hemoglobin concentration, methemoglobin concentration, red blood cell count, hematocrit) and some blood respiratory properties (blood oxygen levels, blood oxygen dissociation curve) when the euryhaline fish, Lates calcarifer is exposed to nitrite in 32 $^{o}/_{oo}$. The present study also aimed at evaluating whether hypoxic conditions occurred in nitrite-exposed fish.

5.3. MATERIALS AND METHODS

5.3.1. Experimental animals

The experimental fish (Lates calcarifer) used in the present experiments were reared from fish fry imported from Thailand. Fish were fed to satiation twice daily with fish powder (Tai Seng Company Ltd., H.K.) which has been made into pellet form by mixing with water.

Fish (90-200 g) were acclimated in 400-l fibreglass tanks containing 32 $^{o}/_{oo}$ sea water for at least 4 weeks before the initiation of the experiments. During the acclimation period and all the subsequent tests, water temperature was maintained at 26-28^{o}C, salinity was 32 $^{o}/_{oo}$ and pH was 7.8-8.0. Oxygen concentration was kept at saturation. Feeding was discontinued 24 hours before fish were transferred into experimental tanks to initiate the experiments.

5.3.2. Hematological parameters and blood respiratory properties

Three tests were conducted. In the first test, the effects of prolonged nitrite exposure on Lates calcarifer were investigated. Seventeen sea bass (145.5 $\pm$ 7.8 g) were randomly selected and divided into treated and control groups. The treated group (n=10) was exposed for 8 days to 10 mgl^{-1} NO_2-N in 400 litres of aerated sea water (32 $^{o}/_{oo}$) while the control group (n=7) was exposed to sea water (32 $^{o}/_{oo}$) with nitrite concentrations less than 1 mgl^{-1} NO_2-N. After an 8 day

immersion period, fish from both groups were removed from the tanks and blood was collected from the severed caudal peduncle. Hematocrit (Hct) was determined immediately after blood collection. Total hemoglobin concentration (Hb) was quantified by the cyanomethemoglobin method (Sigma bulletin No. 525) and red blood cell count (RBC) by counting in a hemocytometer (Neubauer). Methemoglobin concentration (MHb) (as percent of total hemoglobin) was determined by spectrophotometric method outlined by Salvati and Tentori (1981) which consists of measuring changes in absorbance after addition of cyanide to a diluted, hemolysed blood sample. Blood nitrite content was determined by a modification of Azo-dye method proposed by Shechter and Shuval (1972).

The mean corpuscular hemoglobin concentration (MCHC), the mean corpuscular hemoglobin (MCH), and the mean corpuscular volume (MCV) were calculated from the Hct, Hb and RBC by the following formulae (Canong, 1985):

$$\text{MCHC (g 100 ml}^{-1}) = \frac{\text{Hb (g 100 ml}^{-1}) \times 100}{\text{Hct (\%)}}$$

$$\text{MCH (pg)} = \frac{\text{Hb (g 100 ml}^{-1}) \times 10}{\text{RBC (}10^{6}\ \mu\text{l}^{-1})}$$

$$\text{MCV (fl)} = \frac{\text{Hct (\%)} \times 10}{\text{RBC (}10^{6}\ \mu\text{l}^{-1})}$$

A second test was performed to investigate the effects of acute nitrite exposure on Lates calcarifer. Six groups with each consisting of six fishes (95.1 ± 3.2 g) were exposed for 96 hours to sea water (32 $^{o}/_{oo}$) containing 80, 50, 30, 20, 15 or < 1 (control) mgl^{-1} NO_2-N. After 96 hours of different exposures, samples of blood collected from caudal peduncle were analysed for content of hemoglobin, methemoglobin, and blood nitrite. Venous blood oxygen tension (Pvo_2) was also measured.

In the third test, some blood respiratory properties of Lates calcarifer (188.5 ± 6.7 g) after 96 hour exposure to 50 mgl^{-1} NO_2-N were determined. Venous blood was collected from the severed caudal vein whereas arterial blood was sampled by making a cut at the region of dorsal aorta. One point worth mentioning was that L. calcarifer was very sensitive to stress caused by handling so that considerable difficulties were faced in obtaining enough venous and arterial blood from the same fish. Therefore in this experiment, arterial and venous blood were sampled from different batches of fish. The parameters measured were arterial blood oxygen tension (Pao_2), venous blood oxygen tension (Pvo_2), arterial blood oxygen content (Cao_2), venous blood oxygen content (Cvo_2), venous blood pH and total oxygen carrying capacity (Cto_2).

The oxygen dissociation curves of blood from control and treated fish were constructed by the mixing technique (Edwards and Martin, 1966). Blood samples were collected from severed caudal

peduncle and divided into two test tubes containing 0.5 M TRIS buffer at pH 7.8. The blood to buffer ratio was kept constant at 10 : 1. N_2 and air were bubbled through water to obtain gases saturated with water vapour to prevent dehydration of blood samples. Water-saturated air was blown into one portion and humidified N_2 into the other until an equilibrium was reached. The two portions of blood were then mixed in a progressively decreasing proportion of oxygenated blood:deoxygenated blood (i.e. 9:1, 8:2, 7:3 and so forth) in a 100 µl syringe. The Po_2 of the mixed blood samples was then determined by an Oxygen Meter (Model 781, Strathkelvin Instruments). Adjustment of blood pH to 7.8 was made because preliminary study has found that the blood pH of L. calcarifer was roughly equal to this value.

Po_2 was measured with an Oxygen Meter (Model 781, Strathkelvin Instruments) and blood pH was measured with an electrode/analyzer system (Radiometer E5046/PHM71 MK2). Co_2 was determined by the method of Tucker (1967). 20 µl of blood was injected into a chamber containing degassed potassium ferricyanide, which can liberate oxygen into the solution. The oxygen content of the blood can be calculated from the change in the Po_2 of the solution, measured polarimetrically by a Biological Oxygen Monitor (Model 5300, Yellow Spring Instrument Company Ltd.). Cto_2 was measured after the blood was equilibrated with humidified air until there was no further increase in oxygen content.

5.3.3. Statistical analyses

Data were presented as mean ± standard error of mean (SEM). For comparison between two groups, null hypothesis of no difference was tested by Student's t-test. Null hypothesis of no difference between multiple groups was tested by One Way Analysis Of Variance followed by Duncan's Multiple Range Test.

5.4. RESULTS

5.4.1. Changes in hematological parameters in response to prolonged nitrite exposure

Five out of ten fish in the treated group died during the whole experimental period. Apparent gill damage was observed in those dead fish.

The hematological responses of Lates calcarifer after 8 day exposure to 10 mgl^{-1} NO_2-N were shown in Table 5.1. Blood nitrite concentration and methemoglobin content (as percent of total hemoglobin) were significantly increased in treated fish when compared with control fish. On the contrary, treated group showed a significant decrease in the total hemoglobin. Therefore, there resulted in a significant reduction in the functional hemoglobin content in the treated group. No significant difference was found in the value of hematocrit and red blood cell count between the treated and control fish.

Values of hematocrit, red blood cell count and total hemoglobin were used to calculate the three parameters: mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). Calculation showed that MCH and MCHC of the treated group were significantly lower than their respective values of the control group while MCV showed no significant difference between the treated and control fish (Table 5.2)

5.4.2. Changes in hematological parameters and venous blood oxygen tension in response to acute exposure of various nitrite concentrations

Lates calcarifer was exposed for 96 hours to sea water (32 $^{o}/_{oo}$) containing 80, 50, 30, 20, 15 or <1 (control) mgl^{-1} NO_2-N. No mortality was observed during the whole experimental period except when the exposure concentration increased up to 80 mgl^{-1} NO_2-N in which case one out six fish died.

The results of acute exposure were very similar to that of prolonged exposure. Figure 5.1 presents the blood nitrite levels and methemoglobin contents (as % of total hemoglobin) of Lates exposed to various nitrite concentrations. Both blood nitrite concentration and % methemoglobin increased with exposure concentration of nitrite. More than 70 % of total hemoglobin was oxidized to methemoglobin when the exposure concentration increased up to 80 mgl^{-1} NO_2-N. Total hemoglobin concentrations in sea bass declined significantly as external nitrite concentration increased above 50 mgl^{-1} NO_2-N (Table 5.3). A combination of an increase in methemoglobin content and a decrease in total hemoglobin led to a significant reduction in functional hemoglobin in the groups immersed in 30, 50 and 80 mgl^{-1} NO_2-N.

Table 5.4 illustrates the venous blood oxygen tension (Pvo_2) of Lates calcarifer exposed to various nitrite concentrations. Pvo_2 was found to significantly decline in fish exposed to nitrite

concentrations above 30 mgl^{-1} NO_2-N.

5.4.3. Changes in blood respiratory properties in response to acute nitrite exposure

Some blood respiratory properties of Lates calcarifer after 96 hour exposure to 50 mgl^{-1} NO_2-N were investigated. Comparisons of the blood oxygen tension (Po_2), blood oxygen content (Co_2), and blood pH were made between control and treated fish (Table 5.5). Arterial and venous blood oxygen tension (Pao_2 & Pvo_2), arterial and venous oxygen content (Cao_2 & Cvo_2), and blood oxygen carrying capacity (Cto_2) were all significantly lower in treated fish as compared with their respective values in control fish. No significant difference was found in venous blood pH between the treated and control fish.

Whole blood oxygen dissociation curves at pH 7.8 of control and treated fish are presented in Figure 5.2. As shown in the figure, the whole blood oxygen dissociation curve of treated fish has shifted to the left. P_{50} (the oxygen tension when 50 % of the hemoglobin is saturated with oxygen) (Shappell and Lenfant, 1975) for control fish was 14.47 $\pm$ 2.28 mmHg which decreased to 5.93 $\pm$ 1.64 mmHg for treated fish. The Co_2 at 100 % saturation changed from 16.46 $\pm$ 1.72 vols % for control fish to 8.33 $\pm$ 1.35 vols % for treated fish. There was a significant reduction in the total oxygen carrying capacity, indicating a significant Root effect.

Figure 5.3 shows that the Hill coefficient (n) was

significantly changed by nitrite exposure. The values of n are 1.46 and 0.99 for control and treated fish respectively.

Table 5.1. Changes in hematological parameters of *Lates calcarifer* after prolonged (8-day) exposure to 10 mgl^{-1} NO_2-N

	Control (n = 7)	Treated (n = 5)
Hematocrit (%)	31.58 ± 0.82	32.43 ± 2.34
Total hemoglobin (g 100 ml^{-1})	7.72 ± 0.56	4.88 ± 0.51*
Methemoglobin (%)	2.50 ± 0.12	8.35 ± 2.34*
Functional hemoglobin (g 100 ml^{-1})	7.53 ± 0.55	4.51 ± 0.59*
Red blood cell count (10^6 μl^{-1})	3.62 ± 0.40	3.49 ± 0.70
Blood nitrite content (µg NO_2-N ml^{-1})	0.07 ± 0.01	0.34 ± 0.05*

Values are presented as mean ± SEM

*Null hypothesis of no difference between control and treated groups was rejected at $p < 0.05$ (Student's t-test)

Table 5.2. Changes in values of MCV, MCH, and MCHC of red blood cells of *Lates calcarifer* after prolonged (8-day) exposure to 10 mgl^{-1} NO_2-N

	Control (n = 7)	Treated (n = 5)
Mean corpuscular volume (MCV) (fl)	91.48 ± 7.88	104.71 ± 23.24
Mean corpuscular hemoglobin (MCH) (pg)	23.12 ± 3.24	14.96 ± 1.77*
Mean corpuscular hemoglobin concentration (MCHC) (g 100 ml^{-1})	24.72 ± 2.32	15.14 ± 1.40*

Values are presented as mean ± SEM

*Null hypothesis of no difference between control and treated groups was rejected at $p < 0.05$ (Student's *t*-test)

Table 5.3. Hematological responses of Lates calcarifer after acute (96 hour) exposure to various nitrite concentrations

Nitrite concentration (mgl^{-1} NO_2-N)	Number of fish	Hematocrit (%)	Total hemoglobin (g 100 ml^{-1})	Methemoglobin (%)	Functional hemoglobin (g 100 ml^{-1})	Blood nitrite content (μg NO_2-N ml^{-1})
<1 (Control)	6	33.36 ± 2.15^{ab}	8.69 ± 0.49^{ab}	1.82 ± 0.14^{a}	8.54 ± 0.49^{a}	0.06 ± 0.002^{a}
15	6	39.63 ± 5.50^{b}	8.15 ± 0.18^{bc}	15.00 ± 2.26^{b}	6.93 ± 0.71^{ab}	1.92 ± 0.21^{b}
20	6	34.10 ± 0.47^{ab}	8.95 ± 0.88^{bc}	19.65 ± 3.32^{b}	7.22 ± 0.69^{ab}	2.35 ± 0.43^{b}
30	6	31.64 ± 1.75^{a}	8.86 ± 0.16^{b}	34.64 ± 3.70^{c}	5.80 ± 0.37^{b}	3.83 ± 0.19^{c}
50	6	29.92 ± 2.18^{a}	6.97 ± 0.60^{ac}	51.51 ± 4.86^{d}	3.53 ± 0.43^{c}	6.52 ± 0.27^{d}
80	5	29.03 ± 2.38^{a}	6.70 ± 0.75^{c}	73.63 ± 3.11^{e}	2.09 ± 0.67^{c}	13.34 ± 1.08^{e}

Values are presented as mean ± SEM.

Values with different alphabets are significantly different at $p = 0.05$ (One Way Analysis Of Variance followed by Duncan's Multiple Range Test).

Table 5.4. Venous blood oxygen tension of Lates calcarifer after acute (96 hour) exposure to various nitrite concentrations

Nitrite concentration (mgl^{-1} NO_2-N)	Number of fish	Venous blood oxygen tension (mmHg)
<1 (Control)	6	14.06 ± 0.80^a
15	6	12.50 ± 0.52^a
20	6	n.d.
30	6	6.32 ± 0.60^b
50	6	5.65 ± 0.56^b
80	5	4.83 ± 0.49^b

Values are presented as mean $\pm$ SEM.

Values with different alphabets are significantly different at $p = 0.05$ (One Way Analysis Of Variance followed by Duncan's Multiple Range Test).

n.d.: value not determined

Table 5.5. Changes in respiratory properties of the blood of Lates calcarifer after acute (96 hour) exposure to 50 mgl^{-1} NO_2-N

	Control (n = 6)	Treated (n = 6)
Arterial blood oxygen tension (Pao_2) (mmHg)	52.05 ± 1.88	28.39 ± 1.51*
Venous blood oxygen tension (Pvo_2) (mmHg)	14.06 ± 0.80	5.65 ± 0.56*
Arterial blood oxygen content (Cao_2) (vols %)	8.93 ± 1.28	3.32 ± 0.71*
Venous blood oxygen content (Cvo_2) (vols %)	4.59 ± 0.81	1.22 ± 0.19*
Total oxygen carrying capacity (Cto_2) (vols %)	16.46 ± 1.72	8.33 ± 1.35*
Venous blood pH (pHv)	7.84 ± 0.13	7.78 ± 0.11

Values are presented as mean ± SEM

*Null hypothesis of no difference between control and treated groups was rejected at $p < 0.05$ (Student's t-test)

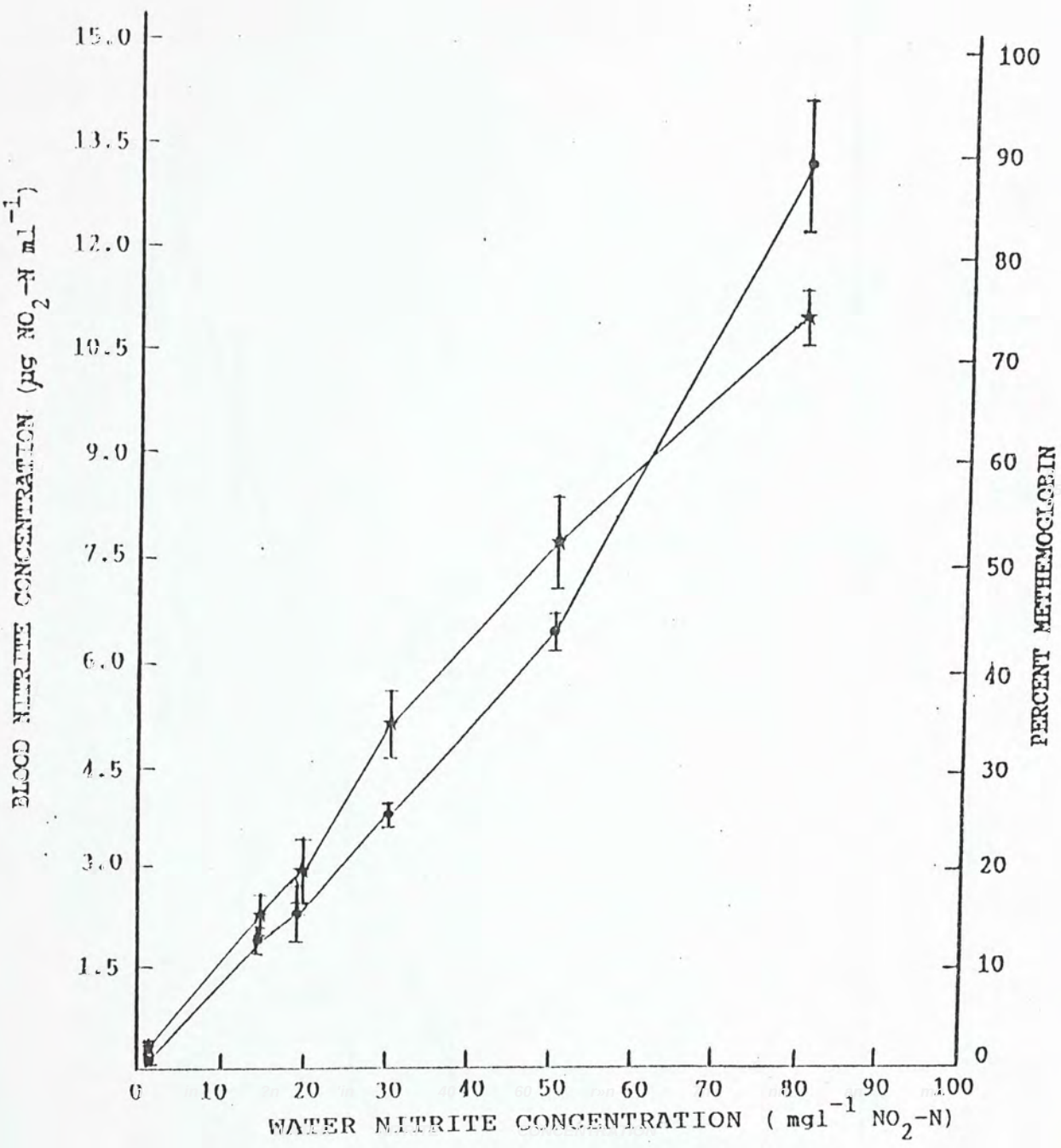

Figure 5.1. Blood nitrite concentration and percent methemoglobin of Lates calcarifer after 96 hour exposure to various nitrite concentrations. Values are mean ± SEM (n = 6). (Blood nitrite concentration: solid circle, Percent methemoglobin: asterisk).

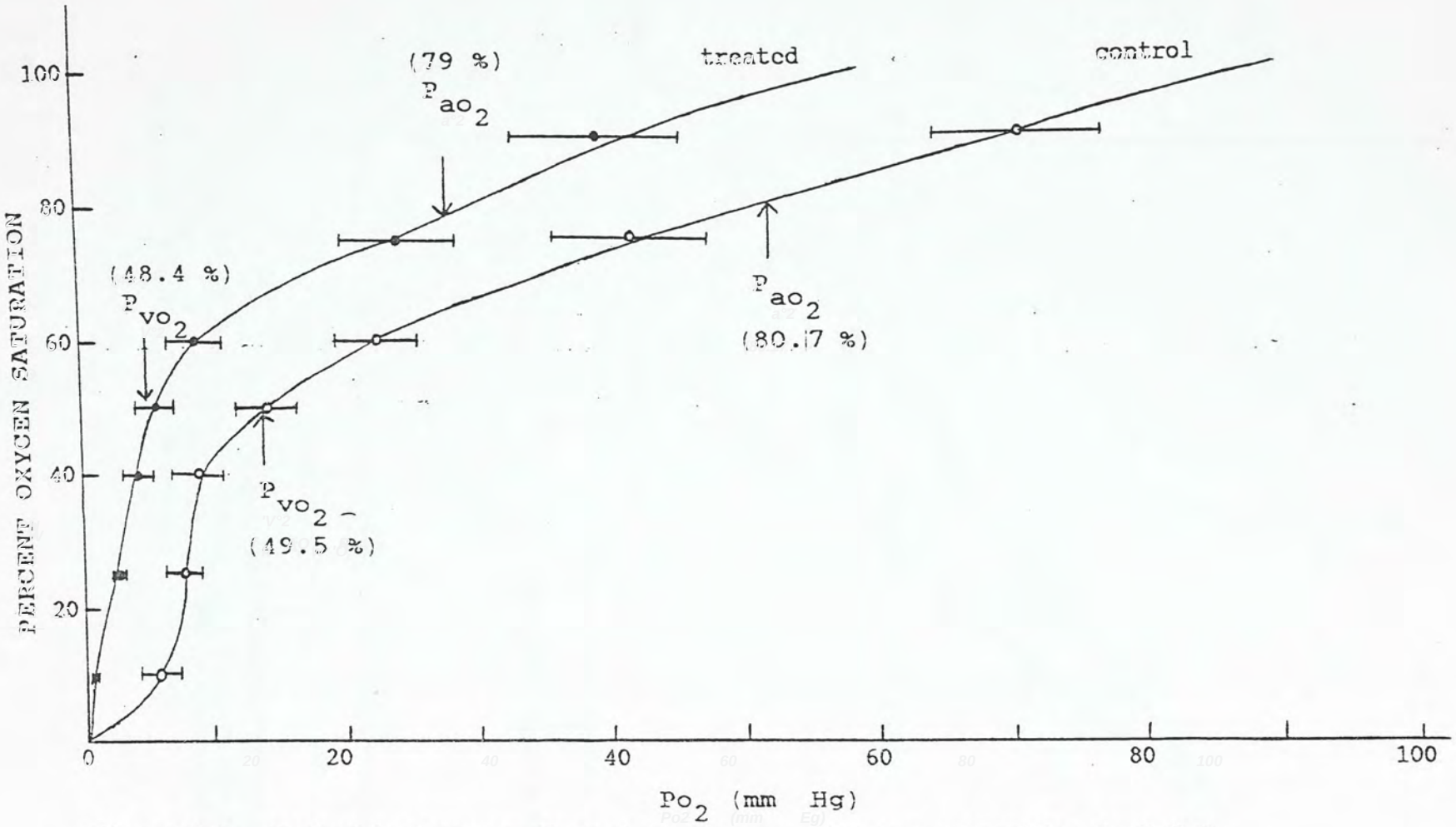

Figure 5.2. Whole blood oxygen dissociation curves of Lates calcarifer at pH 7.8: (o) control, (●) treated (50 mgl^{-1} NO_2-N, 96 hr). The data were obtained at 27 °C in Tris buffer. Values are presented as mean ± SEM, n = 6.

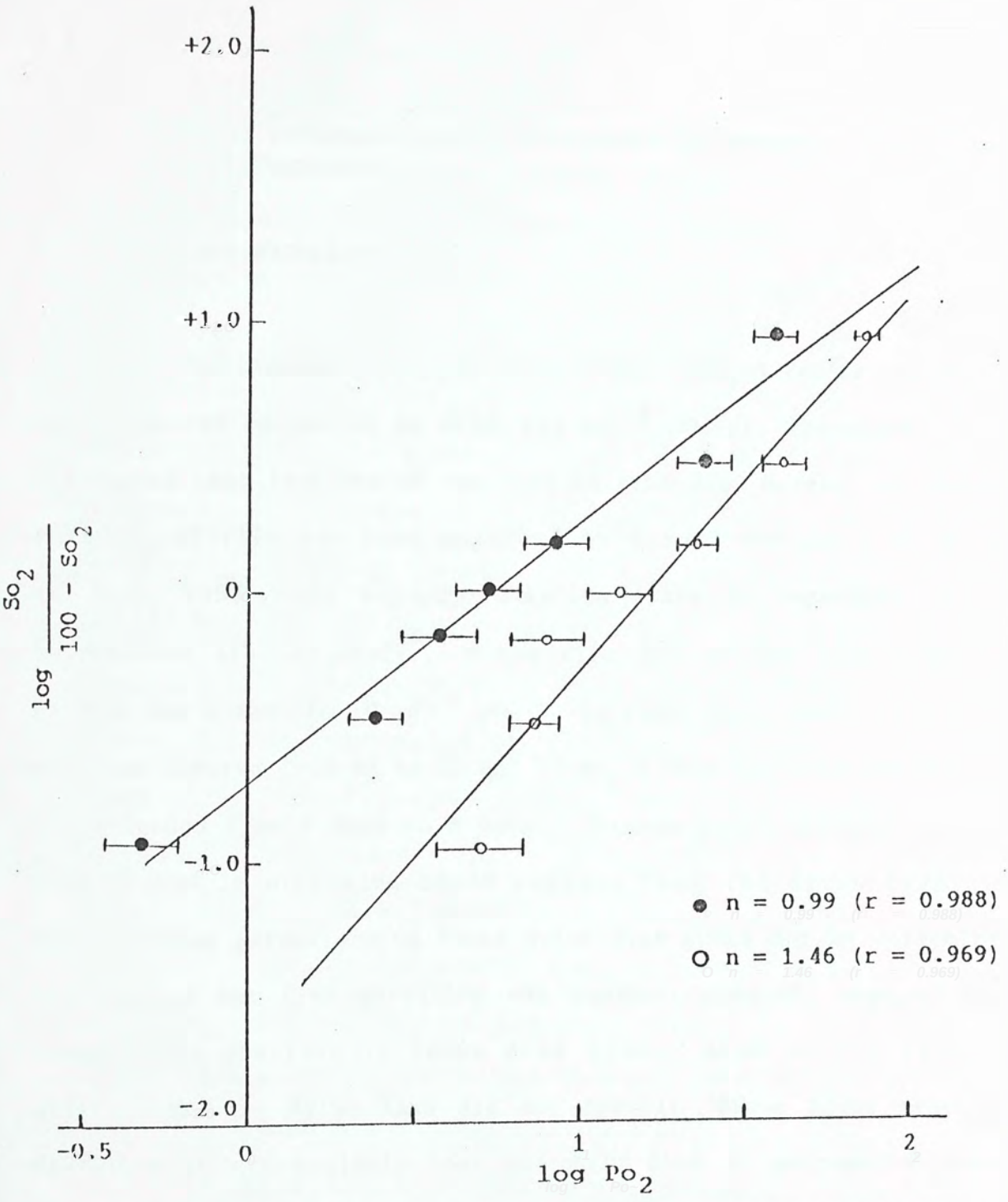

Figure 5.3. The Hill plot of the blood dissociation curves of Lates calcarifer at pH 7.8: (O) control (●) treated (50 mgl^{-1} NO_2-N, 96 hr). n = Hill coefficient and r = correlation coefficient for the lines drawn.

5.5. DISCUSSION

5.5.1. Changes in hematological parameters in response to prolonged nitrite exposure

5.5.1.1. Fish mortality

The exposure concentration 10 mgl^{-1} NO_2-N was relatively low when compared to the 96 hr LC50 (93 mgl^{-1} NO_2-N), therefore, it was unexpected that five out of ten treated fish died during the exposure period. Nitrite has been regarded as a slow-acting fish poison (Klinger, 1957), the exposure duration plays an important role in determining its lethality. Since five out of ten fish died when exposed for 8 days to 10 mgl^{-1} NO_2-N, it might imply that the LC50 was markedly lowered from 93 to 10 mgl^{-1} NO_2-N when the exposure duration was extended from 4 days to 8 days. Because great difficulties were encountered in obtaining blood samples from the dying fish, the hematological parameters of those dying fish could not be determined. The reasons for fish mortality was unclear, however, apparent gill damage were observed in these dead fish. Also by the author's observation, the dying fish did not exhibit "brown blood symptom", therefore it was unlikely that the fish died of methemoglobinemia. Instead, gill failure might be one of the reasons for mortality. It has been reported that nitrite exposure induced gill lesions in *Salmo gairdneri* (Gaino *et als.*, 1983) and *Clarias lazera* (Michael *et al.*, 1986).

5.5.1.2. Methemoglobin formation and total hemoglobin reduction

Methemoglobin, which contains heme iron in Fe^{3+} state, in contrast to hemoglobin, is unable to combine reversibly with oxygen and so cannot be used for oxygen transport (Bodansky, 1951; Bartlett et al., 1987). Methemoglobin forms spontaneously in normal erythrocytes, although slowly, in the absence of nitrite. In mammals, the methemoglobin levels seldom exceed 1 % (Cameron, 1971a). However, under some special circumstances methemoglobin content may rise to a level which is lethal to the organisms. In man clinically important methemoglobinemias can arise under several circumstances. For example, methemoglobinemia is produced by the inheritance of a defect in the erythrocyte methemoglobin reductase enzyme system which normally converts the small amount of methemoglobin continuouslly formed back to hemoglobin (Schwartz et al., 1983). Methemoglobinemia is also produced by the inheritance of so-called "unstable" hemoglobins which are more susceptible than normal to auto-oxidation to methemoglobin (Winslow and Anderson, 1983). Finally, methemoglobinemia in man can result from the ingestion or inhalation of a variety of chemical compounds, including many drugs, notably nitrites (Beutler, 1983). In fish, methemoglobinemia usually results from exposure to high concentrations of nitrite in their natural habitats or culture systems. Unlike mammalian blood, fish blood typically contains a measurable amount of methemoglobin. Reported values for freshwater fish include: 0.9 to 3.6 % for rainbow trout

(Cameron, 1971a; Brown and McLeay, 1975; Smith and Russo, 1975), 10.9 % fo r prespawning pink salmon (Cameron, 1971a), and 2.2 % for channel catfish (Huey et al., 1980). Graham and Fletcher (1986) have assayed the methemoglobin levels in five species of temperate marine teleosts and found that there was at least 7% of total hemoglobin in the methemoglobin-form in all species, and as high as 27 % in one species, the Atlantic cod.

In the present experiment, the methemoglobin level (as percent of total hemoglobin) of seawater-adapted euryhaline Lates calcarifer was found to be 2.50 ± 0.12 %. This value was relatively low when compared to those reported for marine teleosts but comparable to those reported for mammals or freshwater fish such as rainbow trout and channel catfish. The basal level of methemoglobin in L. calcarifer may indicate that this fish species is equipped with an active methemoglobin reductase enzyme system in its red blood cells. Further experiments would be conducted to assay for the activity of this enzyme (See Section 6.3).

When the sea bass were exposed to the test water, nitrite ions enter the fish body either through the passive diffusion of nitrous acid (Eddy et al., 1983) or through the anion exchange mechanism in the lamellar chloride cells (Bath and Eddy, 1980; Meade and Perrone, 1980; Krous et al., 1982) or both. It is well known that chloride protect fish against nitrite toxicity (Crawford and Allen, 1977; Perrone and Meade, 1977). Chloride competes with

nitrite for transport across the gills (Crawford amd Allen, 1977; Perrone and Meade, 1977; Russo and Thurston, 1977) and thus inhibiting nitrite uptake by the fish. As shown in Table 5.1, blood nitrite concentration of treated fish was found to be 5 fold of that of control fish. However, unlike most freshwater fish in which blood nitrite levels may reach up to 10 times (Bath and Eddy, 1980) or even 60 times (Margiocco et et., 1983) the nitrite concentrations of the medium, the nitrite concentrations in blood of treated Lates calcarifer never exceeded that of test water. Since 32 $^{o}/_{oo}$ sea water was employed in this study, it was expected that the high chloride concentration in the test water could somewhat inhibit nitrite uptake by the fish and thus preventing the strong bioaccumulation of nitrite in the blood as usually occurred in freshwater fish.

Nitrite in the fish blood readily oxidizes the heme iron of hemoglobin to form methemoglobin. Because blood nitrite concentration in treated fish was only 0.34 μg NO_2-N ml^{-1} blood, a relatively small, though significant, increase in percent methemoglobin was observed.

On the other hand, there was a significant reduction in the total hemoglobin content in treated fish when compared with control fish. Reduction in total hemoglobin content induced by nitrite exposure has also been reported for freshwater fish Clarias lazera (Hilmy et al., 1987) and seawater fish Dicentrarchus labrax (Scarano et al., 1984). As a result of a decrease in total hemoglobin content

and an increase in methemoglobin level, the treated fish demonstrated a significant decrease in functional hemoglobin concentration. Decrease in total hemoglobin content may be caused by hemolysis of red blood cells since it has been reported by Scarano et al. (1984) that histochemical analysis of spleen tissues from fish exposed to nitrite showed Fe^{3+} originating from hemoglobin destruction and indicated hemolytic anemia caused by spleen macrophages. If there was hemolysis of red blood cells, erythrocyte count and hematocrit value should have been decreased, but they were not. The present study found that erythrocyte count and hematocrit value did not change significantly. This indicated that decrease in total hemoglobin was not due to decrease in red blood cell volume as seen from the unchanged value of MCV. The observed decrease in total hemoglobin must have been the result of a true reduction in the hemoglobin content of each red blood cell. This interpretation was supported by a significant decrease in the values of MCH and MCHC.

Methemoglobin formation was the most consistent hematological responses so far reported when fish were exposed to nitrite. Other hematological responses such as total hemoglobin, hematocrit and red blood cell count varied considerably among different studies (See Section 2.4.1.2.). It is likely that the hematological responses to nitrite exposure depend on many factors such as species-specificity, environmental conditions and nutritional conditions of the fish. The mechanism by which nitrite reduces the total hemoglobin content in fish is unknown and no studies have been

directed to this aspect. Short supply of iron has been proposed as a possible cause of hemoglobin deficiency (Cameron and Wohlschlah, 1969). However, no studies have been devoted to investigate the effect of nitrite on the supply or utilization of iron in fish.

Oxygen transport depends upon a number of factors. The concentration of hemoglobin is one of them. Total hemoglobin level of an animal can reflect its respiratory requirement. As the respiratory requirements of animals vary over an enormous range, the total hemoglobin levels vary concomitantly. At one extreme, the metabolic requirements for oxygen are so low that an oxygen transport pigment is unnecessary: sufficient oxygen is physically dissolved (Riggs, 1972). Therefore the antarctic ice fish which live in cold highly oxygenated waters and are of sluggish habits obviate the need for a respiratory pigment (Cameron and Wohlschlag, 1969). At the other extreme, metabolically active fish often have such large requirements for oxygen that blood hemoglobin is not only necessary but even a slight anemia may be serious disadvantage (Riggs, 1972). Cameron and Wohlschlag (1969) studied the respiratory response of the pinfish to experimentally induced anemia. They found that the resting metabolism of the fish showed no significant difference related to hemoglobin level, though anemic fish were slightly higher, and the cardiac rates showed a compensatory increase in response to hemoglobin reduction which accounted for about 50 % of the predicted increase in cardiac output. Concerning the role of hemoglobin, the authors

concluded that "Hemoglobin allows the circulatory system under ordinary conditions to operate at a much lower level of energy expenditure than it could with it, thus providing a considerable reserve for extra demands made by activity or other stress. Reduction or inactivation of hemoglobin merely pushed the level of circulatory operation up closer to the limits, so that the reserve is considerably reduced". On this basis, it is reasonable to speculate that the lowering hemoglobin levels caused by nitrite exposure rendered the fish an immediate effect of increasing the maintenance metabolism requirements (Cameron and Wohlschlag, 1969). In order to meet this stress either the feeding rate would have to be increased or the growth rate slowed correspondingly. The second effect of lowered hemoglobin level is a restriction of activity levels.

Much literature describes that fish respond to hypoxia by an increase in hematocrit and hemoglobin concentration (Wood et al., 1975; Smit and Hattingh, 1978b). However, there is always no corresponding increase in the red blood cell count. Therefore some researchers proposed that the typical rise in hematocrit and hemoglobin concentration seen during hypoxia was not due to new erythrocyte synthesis (Cameron, 1971) but due to hemoconcentration (i.e. water loss) and swelling of the red blood cell (Albers, 1970). Although nitrite has long been regarded as a strong chemical to induce hypoxia, the hematological parameters of Lates calcarifer exposed for 8 days to nitrite did not reveal any hypoxic symptoms. The decrease in the value of total hemoglobin observed during this study is due to

the toxic effect of nitrite itself rather than hypoxia.

5.5.2. Changes in hematological parameters and venous blood oxygen tension in response to acute exposure of various nitrite concentrations

5.5.2.1. Methemoglobin formation and total hemoglobin reduction

Blood nitrite levels increased more or less proportionally with the test concentrations of nitrite at the range of 15 to 50 mgl^{-1} NO_2-N. Blood nitrite level of fish exposed to 80 mgl^{-1} NO_2-N was nearly 2 fold of that of fish exposed to 50 mgl^{-1} NO_2-N. It might be that when fish were exposed to such a high concentration of nitrite, those barrier such as the integument which initially restricted the entry of nitrite was damaged so that nitrite could flux into the fish body more easily. As shown in Figure 5.1, percent methemoglobin was highly correlated ($r = 0.994$) with the exposure concentration of nitrite. Percent methemoglobin reached more than 70 % as exposure concentrations increased up to 80 mgl^{-1} NO_2-N. Only 25 % of total hemoglobin remained as functional hemoglobin to transport oxygen. This low functional hemoglobin level should confer considerable stress on the cardiac energy output of the fish as suggested by Cameron and Wohlschlag (1969). The 96 hr LC50 of nitrite for Lates calcarifer in 32 $^{o}/_{oo}$ was found to be 93 mgl^{-1} NO_2-N in the previous study. In Figure 5.1, the percent methemoglobin level at the 96 hr LC50 was estimated to be 83 % which probably represented a lethal level for L.

calcarifer. The total hemoglobin decreased significantly as external nitrite concentrations increased up to 80 mgl^{-1} NO_2-N. Prolonged exposure of fish to 10 mgl^{-1} NO_2-N for 3 days resulted in a reduction in the total hemoglobin, however, no such decrease was observed in fish exposed for 4 days to higher concentrations of nitrite (15, 20, 30 and 50 mgl^{-1} NO_2-N). This reflects that nitrite may require a longer time to exert its effect on those physiological processes that regulate the synthesis of hemoglobin. The hematological parameters of L. calcarifer exposed to such high nitrite concentrations also did not reveal any hypoxic symptoms. One possible reason is that L. calcarifer is a species that is very tolerant to hypoxia. Wu (1988) have demonstrated that L. calcarifer could survive without any stress symptoms for 7 hours when subject to 1 mg O_2l^{-1}. Biochemical analysis of metabolites of nitrite-exposed sea bass can provide additional information on the evaluation of whether nitrite exposure induces hypoxia in the fish. This aspect will be discussed in the next chapter.

5.5.2.2. Venous blood oxygen tension

Treated sea bass except those subject to 15 mgl^{-1} NO_2-N exhibited a significant reduction in their venous blood oxygen tension. There are two implications for that. The first is that the blood oxygen content of treated fish is really lowered. The second is that the blood oxygen content is not lowered, but the metabolism is

pumped up due to nitrite exposure so that more oxygen is depleted from the blood stream, thus resulting in a lowered venous blood oxygen tension in treated fish.

5.5.3. Changes in blood respiratory properties in response to acute nitrite exposure

5.5.3.1. Blood oxygen dissociation curve

It is generally accepted that the shape of whole blood dissociation curve determines the range of oxygen tensions for loading and unloading oxygen. The shape of the curve and its slope in the middle range segment are empirically defined by the coefficient n of the Hill equation:

$$S = \frac{100KP^n}{1 + KP^n}$$

where P is oxygen pressure in mmHg, S is oxyhemoglobin saturation and K is a constant (Shappell and Lenfant, 1975). The normal human blood dissociation curve is sigmoid shape which results from the heme-to-heme interactions within the hemoglobin molecule. The normal range of n is 2.6-3.0 (Shappell and Lenfant, 1975). The position of the curve P_{50} is defined as the oxygen tension when 50 % of the hemoglobin is saturated with oxygen. P_{50} is used as an index of the hemoglobin affinity for oxygen. In physiological terms, affinity is inversely related to the pressure of oxygen required to assure a given oxygen saturation. For instance, if the <u>in vitro</u> P_{50} is lower than the

normal value, the hemoglobin affinity for oxygen is greater than normal (Shappell and Lenfant, 1975). A shift of the blood dissociation curve to the right or to the left of its normal position could affect the delivery of oxygen to the tissues. For the same end arterial oxygen tension, a rightward shift yields a lower oxygen saturation at the venous level and thus a wider arteriovenous difference in oxygen saturation (content). This will enhance oxygen delivery, or maintain oxygen delivery while sparing myocardial work. On the contrary, a leftward shift reduces oxygen delivery, or else causes an increase in cardiac output (Shappell and Lenfant, 1975).

The blood oxygen dissociation curve at pH 7.8 of normal Lates calcarifer is rather hyperbolic, with a Hill coefficient (n) equals to 1.46 (Figure 5.2.). This is comparable to the generally unsigmoidal curve of fishes (Grigg, 1974). However, active species like trout (Cameron, 1971b) usually display a sigmoidal curve with n = 2.5-3.0, which is very similar to that of mammals. More sluggish species like carps (Black, 1940) are characterized by a more hyperbolic curve with n = 1.2-1.5 (Grigg, 1974). In species with a monomeric hemoglobin (for example, the hagfish Eptatretus stoutii), the curve assures a typical hyperbolic shape and n value of approximately 1 (Shappell and Lenfant, 1975). The trend that sluggish fish exhibit a more hyperbolic curve while active fish display a sigmoidal curve is in agreement with our study because L. calcarifer ia a very sluggish species with restricted activity levels.

Lates calcarifer blood has a P_{50} value of 14.47 ± 2.28 mmHg which is comparable to the respective value (17 mmHg) of an related freshwater species L. albertianus (Fish, 1956). L. calcarifer has a relatively high blood oxygen affinity as compared with those active fish such as trout (Cameron, 1971b). However, a comparison with the dissociation curves obtained from other fish which survive well in poorly aerated water such as Channa maculata (Yu and Woo, 1987), Tilapia esculenta (Fish, 1956), and Clarias mossambicus (Fish, 1956) shows that the hemoglobin in L. calcarifer blood has a relatively low affinity for oxygen. The competence of the L. calcarifer hemoglobin as an oxygen collector is low, but as a result, the gas is more readily released in the body. Although Lates has a relatively low blood oxygen affinity, it can survive well in water of dissolved oxygen as low as 1 mg O_2 l^{-1} (Wu, 1988). When exposed to hypoxic conditions, fish may regulate their blood oxygen affinity by changing the blood pH or the organo-phosphate (ATP) levels in erythrocytes. It has been well documented that increasing blood pH or decreasing the organo-phosphate levels in erythrocytes can raise the blood oxygen affinity which enable an easier loading of oxygen at gills when the oxygen level of surrounding water is low (Riggs, 1975; Smit and Hattingh, 1981a).

The shape of the blood oxygen dissociation curve of treated Lates calcarifer becomes more hyperbolic with a Hill coefficient (n) of 0.99. A comparison of the dissociation curves of treated and control fish reveals that nitrite exposure shifts the dissociation

curve to the left. Similar displacement of dissociation curve due to conversion of hemoglobin to methemoglobin by nitrite has also been reported in other fish (eg. Bartlett, et al., 1987). The P_{50} for nitrite treated fish was 5.93 ± 1.64 mmHg. This value is comparable to those reported for fish species which can survive well in water of low oxygen levels (Fish, 1956). The low P_{50} in treated fish indicated that the blood of treated fish had a high affinity for oxygen.

5.5.3.2. Blood oxygen tension and oxygen content

When fish were exposed to 50 mgl^{-1} NO_2-N, nearly 50 % of total hemoglobin was converted to methemoglobin which was incapable of carrying oxygen. Conversion of hemoglobin to methemoglobin greatly decreases the oxygen carrying capacity. As shown in Table 5.4., the blood oxygen carrying capacity was significantly decreased from 16.46 vols % in control fish to 8.33 vols % in treated fish. The percent decrease was about 50 % which was in line with the percent conversion of hemoglobin to methemoglobin. In order to compensate for the decrease in oxygen carrying capacity, treated fish have to increase their blood oxygen affinity to facilitate the loading of water oxygen at the respiratory surfaces.

Higher blood oxygen affinity may hinder the loading of oxygen from blood capillaries to tissues. Pao_2 of control fish is 52.05 mmHg (Table 5.4.) which represents 80.7 % oxygen saturation of hemoglobin

while Pao_2 of treated fish is 28.39 mmHg which represents 79 % oxygen saturation of hemoglobin. Pvo_2 of control fish equals to 14.06 mmHg which occupies the position of 49.5 % of oxygen dissociation curve while Pvo_2 of treated fish is 5.65 mmHg which occupies a simlar position of 48.4 % of oxygen dissociation curve. The similar position of Pao_2 and Pvo_2 of treated and control fish at their oxygen dissociation curves implies that the oxygen delivery tendency from blood capillaries to tissues are not significant different between treated and control fish. The exact arteriovenous oxygen difference ($Cao_2 - Cvo_2$) cannot be calculated since the arterial and venous blood were sampled from two different batches of fish. However, the data (Table 5.4.) more or less reflect that treated fish have a lower arteriovenous oxygen difference which means a lower oxygen utilization. In conclusion, although nitrite exposure resulted in a significant reduction in blood oxygen carrying capacity and a significant increase in blood oxygen affinity, there was no evidence however that oxygen transport and delivery are impaired. To the best knowledge of the author, no literature has given direct evidence that oxygen transport and delivery are impared in nitrite-exposed fish.

5.5.3.3. Venous blood pH

The unchanged blood pH value (Table 5.4.) of treated fish when compared with control fish suggests that hypoxia unlikely occurred in them even at a concentration of 50 mgl^{-1} NO_2-N (half of

the 96 hr LC 50). If hypoxia did occur, the lactate produced should lower the blood pH. However, it cannot preclude the possibility that the treated fish did produce considerable amount of lactic acid in the blood because of hypoxia but that the lactate was neutralized by the elevated amount of ammonia in blood. Therefore, the undetectable changes in blood pH *per se* cannot lead to a firm conclusion that hypoxia did not occur in the treated fish. Additional information is required for drawing a conclusion on this aspect.

CHAPTER SIX METABOLIC CHANGES OF *LATES CALCARIFER* IN RESPONSE TO ACUTE AND PROLONGED NITRITE EXPOSURE

6.1. SUMMARY

1. Metabolic responses of Lates calcarifer to acute and prolonged nitrite exposure were studied.

2. Prolonged (8 day) exposure to 10 mgl^{-1} NO_2-N caused an increase in serum sodium, potassium and ammonia concentrations, and a decrease in lipid content. On the other hand, no significant change was found in the tissue composition of liver and muscle.

3. Acute (96 hour) exposure to 30, 50 and 80 mgl^{-1} NO_2-N resulted in a decrease in serum protein concentration, and an increase in serum lactate, ammonia and urea contents. Metabolic changes in serum were most evident when nitrite concentration increased up to 80 mgl^{-1} NO_2-N. Decreases in liver glycogen and muscle lactate were also observed.

4. Fish subject to acute (96 hour) exposure of 50 mgl^{-1} NO_2-N showed decreases in branchial Na^+-K^+-activated adenosine triphosphatase (Na^+-K^+-ATPase), hepatic glutamate-oxaloacetate transaminase (GOT), and glutamate dehydrogenae (GLDH) activities. Contents of liver adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) were all reduced in nitrite treated fish.

5. L. calcarifer was found to possess the enzyme NADH-

methemoglobin reductase which is capable of converting methemoglobin back to hemoglobin. However, acute or prolonged nitrite exposure did not induce changes in its activity.

6. It was concluded that nitrite exposure did not trigger remarkable metabolic symptoms of hypoxia in Lates unless the test concentration reached as high as 80 mgl^{-1} NO_2-N. However nitrite exposure induced an increase in production and excretion of the nitrogenous product, ammonia, as reflected by the elevated level of ammonia in serum. In case of extreme high nitrite intoxication, Lates resorted to produce an uncommon fish nitrogenous product, urea, in addition to ammonia.

6.2. INTRODUCTION

Despite the well documentation of the hematological responses of freshwater fish to nitrite exposure (see Section 2.4.1), limited studies have been devoted to investigate the metabolic changes of nitrite-treated fish. One detailed study carried out by Arillo et al. (1984) found that freshwater fish respond by decreases in blood glucose, liver glycogen and ATP, and increases in liver and brain lactate during advanced stages of nitrite intoxication. Other metabolic effects reported include reduction in serum protein level (Hilmy et al., 1987), in vitro inhibition of branchial carbonic anhydrase activity (Gaino et al., 1984), and increases in serum glutamate oxaloacetate and glutamate pyruvate transaminases activities (Michael et al., 1986). Due to the conversion of hemoglobin to methemoglobin, a form which is incapable of carrying oxygen, some authors (eg. Huey et al., 1980; Hilmy et al., 1987) have proposed that heavy nitrite loads can confer hypoxia or anoxia in fish. Typically, fish respond to hypoxia by decreases in liver glycogen and ATP, and increases in liver lactate, α-glycerophosphate and succinate (Jorgensen and Mustafa, 1980a, b; Yu and Woo, 1987). Elevated serum Na^+, K^+ and Ca^{2+} concentrations were also observed when fish were exposed to extremely hypoxic conditions (1 mg $O_2 l^{-1}$) (Woo and Wu, 1984). Since not many studies have been directed towards elucidating the metabolic effects of nitrite on fish, whether nitrite-treated fish exhibit metabolic derangements in response to hypoxia is still subject

to argument.

Since the metabolic responses of euryhaline fish to nitrite exposure have not been studied, it is the objective of this study to compare the metabolic parameters (serum ions, blood metabolites such as glucose, protein and lactate, liver and muscle metabolites such as glycogen, protein, lipid and water content, and liver enzyme activities) when the euryhaline fish Lates calcarifer is exposed to nitrite. The present study also aims at evaluating whether hypoxic conditions occurs in nitrite-exposed fish.

Mammals including humans normally contain the NADH-methemoglobin reductase to reconvert methemoglobin to hemoglobin (Jaffe, 1964; Cameron, 1971a). However, this enzyme system does not universally occur in fish (Freeman et al, 1983). Therefore, the present study is also devoted to determine whether Lates calcarifer possesses this enzyme.

6.3. MATERIALS AND METHODS

6.3.1. Experimental animals

The experimental fish (Lates calcarifer) used in the present experiments were reared from fish fry imported from Thailand. For experiments which studied the effects of prolonged nitrite exposure, fish bought from the local market were also employed. Fish were fed to satiation twice per day with fish powder (Tai Sang Company Ltd., H.K.) which has been made into pellet form by mixing with water. Fish were acclimated in 400-l fibreglass tanks containing 32 $^{o}/_{oo}$ sea water for at least 4 weeks before the initiation of the experiments. During the acclimation period and all the subsequent tests, water temperature was maintained at 26-28 ^{o}C, salinity was 32 $^{o}/_{oo}$ and pH was 7.8-8.0. Oxygen concentration was kept at saturation. Feeding was discontinued 24 hours before fish were transferred into experimental tanks to initiate the experiments.

6.3.2. Experimental protocols

Five tests were conducted in the present study. In the first test, two trials have been carried out to investigate the effects of prolonged nitrite exposure on the metabolite contents in serum, liver and muscle of Lates calcarifer. In the first trial, twelve fish (1009.5 $\pm$ 28.6g) bought from local market were randomly divided into treated and control groups. The treated group (n = 6) was exposed to 10 mgl^{-1} NO_2-N for 8 days in 400 litres of aerated sea

water while the control group (n = 6) was exposed to sea water with nitrite concentration less than 1 mgl^{-1} NO_2-N. After an 8 day immersion period, fish from both groups were removed from the tanks and blood was rapidly collected from the severed caudal peduncle. Whole blood was allowed to clot at room temperature for 45 minutes and then centrifuged to obtain serum. The entire liver was excised to obtain the hepatosomatic index (liver weight x 100 % / body weight). The liver, brain and a piece of epaxial muscle were rapidly collected and frozen. All samples were subsequently frozen at -20°C until further analysis. In the second trial, the fish employed were the same as those used for hematological studies in Chapter 5 (See Section 5.3.2, 1st paragraph). The experimental procedure was the same as described above.

A second test was carried out to investigate the effects of acute nitrite exposure on the metabolite contents of Lates calcarifer. The fish employed were the same as those used for hematological studies in Chapter 5 (See Section 5.3.2., 2nd paragraph). Four groups of fish were exposed to sea water containing 80, 50, 30 or <1 (control) mgl^{-1} NO_2-N. After subject to different exposures for 96 hours, whole blood collected from caudal peduncle was centrifuged to obtain serum. The whole liver, brain and a piece of epaxial muscle were rapidly removed and subsequently frozen at -20 °C until further analysis.

In the third test, branchial Na^+-K^+-ATPase, hepatic ATP,

ADP and AMP concentrations, and some hepatic enzyme activities of Lates calcarifer (112.7 ± 6.7g) exposed to 50 mgl^{-1} NO_2-N for 96 hours were determined. Twelve fish were randomly selected and divided into control and treated groups. The treated group was immersed in sea water (32 $^{o}/_{oo}$) containing 50 mgl^{-1} NO_2-N whereas the control group was exposed to sea water. After a 96 hour immersion period, the fish of both groups were sacrificed, the gill and liver were quickly removed and put into liquid nitrogen. All samples were subsequently frozen at -20 ^{o}C until analysis.

The fourth test was undertaken to determine the effect of nitrite exposure on the rate of ammonia excretion of Lates calcarifer. Fish were randomly divided into treated and control groups. Control group (205.1 ± 5.3 g) was immersed in sea water (32 $^{o}/_{oo}$) while the treated group (208.0 ± 5.0 g) was exposed 96 hours to sea water containing 50 mgl^{-1} NO_2-N. After that, fish in both groups were transferred into nitrite-free sea water of which the ammonia content has been previously measured. At certain time intervals, water samples were taken for the analysis of ammonia content. The average rates of ammonia excretion for both groups were then calculated.

The final test was conducted to assay the blood NADH-methemoglobin reductase activity of Lates calcarifer. The test also aimed at evaluating whether acute or prolonged nitrite exposure had effects on the activity of this enzyme. Fish (180.6 ± 8.4 g) were divided into three groups. The first group was subject to acute (96

hour) exposure of 50 mgl^{-1} NO_2-N, the second group was subject to prolonged (8 day) exposure of 10 mgl^{-1} NO_2-N while the last group served as the control. After 96 hours, half of the control fish and those from the acute exposed group were killed and blood was collected from the severed caudal peduncle. The blood samples were used immediately for the determination of NADH-methemoglobin reductase. By the end of the 8 day immersion period, the remaining control fish and those from prolonged exposed group were sacrificed and the blood samples collected from the severed caudal peduncle were immediately assayed for the enzyme.

6.3.3. Chemical and biochemical analyses

6.3.3.1. Metabolite and electrolyte contents

Serum sodium, potassium and calcium concentrations were determined using flame photometry (ISA Biologie) after appropriate dilution of the serum samples. Serum chloride concentration was determined using a Corning Chloride Meter. Serum glucose was estimated using a coupled glucose oxidase-peroxidase reaction according to Sigma Procedure (No. 510). Serum lactate level was estimated using lactate dehydrogenase following the method of Hohorst (1962). Serum protein content was determined according to Hartree (1972), total lipids according to the sulphurophosphovanillin method of Woodman and Price (1972), cholesterol using enzymes cholesterol oxidase and cholesterol esterase, coupled with 4-aminoantipyrine

chromogenic system according to Sigma Procedure (No.351), and free amino acids according to the ninhydrin reaction as described by Matthews et al. (1964). Serum ammonia level was determined using glutamate dehydrogenase for the reductive amination of 2-oxoglutarate according to Sigma Procedure (No. 170-UV) and serum urea was determined by the method outlined by Hunninghake and Grisolia (1966).

Liver and muscle samples were dried at 100 ^{o}C to constant weight and then defatted using petroleum ether (Searle, boiling range 60-80 ^{o}C). The defatted samples were weighed and the percentages of water and lipid in the tissues were calculated using weight differences.

Tissue samples were homogenized in distilled water (1:10; w/v) using an Ultra-Turrax Homogenizer. The whole homogenate was used to determine tissue glycogen (Murat and Serfarty, 1974) and protein (Hartree, 1972) concentrations. For the determination of tissue lactate content, tissues were homogenized in 1 M perchloric acid and the supernatant was used for the determinations. To quantify the content of liver ATP, frozen liver powder was homogenized in 6 % perchloric acid and the ATP content of the supernatant was determined with hexokinase and glucose-6-phosphate according to the method of Lamprecht and Trautschold (1974). To estimate the liver ADP and AMP contents, frozen liver powder was homogenized in 0.9 M perchloric acid. The ADP and AMP concentration of the supernatant was estimated by using pyruvate kinase, lactate dehydrogenase and myokinase as

described by Jaworek (1974). The adenylate energy charge was calculated from the ATP, ADP and AMP levels by the following formula (Atkinson, 1968):

$$\text{adenylate energy charge} = \frac{\text{ATP} + 1/2\ \text{ADP}}{\text{ATP} + \text{ADP} + \text{AMP}}$$

6.3.3.2. Branchial Na^+-K^+-ATPase activity

Gill filaments were homogenized in cold solution containing 0.3 M sucrose, 1 mM EDTA and 0.1 % sodium deoxycholate at pH 7.4 using a Ultra-Turrax Homogenizer. Na^+-K^+-ATPase activity was measured in the whole homogenate in the standard incubation medium: 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2.6H_2O$, 5 mM Na_2ATP and 40 mM Tris-HCl buffer at pH 7.4. The reaction was allowed to proceed at 25 ^oC for 15 min and stopped by the addition of 2 ml cold 10 % trichloroacetic acid. Inorganic phosphate liberated from ATP was determined according to Harper (1965). Protein concentration of the gill homogenate was determined according to Hartree(1972).

6.3.3.3. Hepatic enzyme activities

For enzyme determination, a portion of liver weighing about 100 mg was homogenized in 10 volumes of physiological saline (0.8 % NaCl). Enzymes in the homogenate were studied at 25 ^oC.

Glycogen phosphorylase a (phosphorylase) activity was assayed as described by Ummlnger and Benziger (1975). Activities of glucose-6-phosphatase (G6Pase) was determined according to Baginski et al. (1974) while glutamate dehydrogenase (GLDH) according to King (1974). Lactate dehydrogenase (LDH), in the direction of pyruvate to lactate, was assayed according to Bergmeyer and Bernt (1974). Assays of isocitrate dehydrogenase (ICDH) and glucose-6-phosphate dehydrogenase (G6PDH) activities were carried out as described by Chan and Woo (1978). Glutamate-oxaloacetate transaminase and glutamate-pyruvate transaminase activities were assayed according to Sigma Procedure (No. 505).

6.3.3.4. Blood NADH-methemoglobin reductase activity

NADH-methemoglobin reductase activity was determined by the method of Scott (1960). Blood samples were centrifuged to obtain red blood cells. The cells were washed with phosphate-buffered saline solution (9 volumes 0.9 % NaCl, 1 volume 0.1 M potassium phosphate, pH 7.3). To the cells was added an equal volume of phosphate-buffered saline solution containing 1 % sodium nitrite and the mixture was shaken and allowed to stand for 20 minutes. The cells were then washed with phosphate-buffered saline solution to completely remove the nitrite. The oxidized red blood cells were lysed by addition of water containing 0.05 % Triton X-100 which was used for complete solubilization of the enzyme (Scott and Harrington, 1985). The

methemoglobin of the hemolysate was estimated by measuring absorption at 600 nm.

Hemolysate equivalent to a fixed amount of methemoglobin was added to a reaction mixture containing 1 M Tris at pH 7.6, 10 mM EDTA and 1.2 mM DCIP (2,6-dichlorophenolindophenol, a dye that causes fluorescence in the presence of NADH). Water was added instead of hemolysate as the control. If the methemoglobin reductase system is present in the blood, the reaction mixture produces NADH-methemoglobin reductase that converts the methemoglobin to hemoglobin. NADH oxidizes to NAD, which does not fluoresce. Therefore the enzyme activity can be estimated by measuring the decrease in absorbance corrected for the blank. Activity measurements were carried out at 23 ^{o}C.

6.3.3.5. Water ammonia content

Ammonia content in water is determined by phenolhypochlorite method according to Strickland and Parsons (1972).

6.3.4. Statistical analyses

Data are presented as means $\pm$ standard error of mean (SEM). For comparison between two groups, null hypothesis of no difference was tested by Student's t-test. Null hypothesis of no difference between multiple groups was tested by One Way Analysis Of Variance followed by Duncan's Multiple Range Test.

6.4. RESULTS

6.4.1. Changes in metabolite content in response to prolonged nitrite exposure

The results obtained in the two trials were grouped together. Table 6.1 presents the serum Na^+, K^+, Ca^{2+} and Cl^- levels of Lates calcarifer after 8 day exposure to 10 mgl^{-1} NO_2-N. Both serum Na^+ and K^+ concentrations in treated fish were significantly higher than that of control fish. No significant difference was found in serum Ca^{2+} and Cl^- levels between treated and control fish.

As shown in Table 6.2, serum lipid significantly declined in fish exposed to nitrite but serum glucose, lactate, protein, cholesterol, α-amino acid and urea concentrations were unchanged. On the contrary, serum ammonia level was markedly elevated upon exposure to nitrite. Tissue composition of liver, muscle, and brain did not show any significant change (Table 6.3)

6.4.2. Changes in metabolite content in response to acute exposure to various nitrite concentrations

There was a clear trend of increasing serum lactate concentration as Lates calcarifer were exposed to nitrite (Table 6.4). The level was unproportionally high as test concentration increased up to 80 mgl^{-1} NO_2-N. Serum protein levels were significantly lowered only when the fish were subject to 80 mgl^{-1} NO_2-N. Both serum ammonia and urea contents were markedly elevated in nitrite-treated fish.

Table 6.5 shows the tissue composition of Lates calcarifer after 96 hour exposure to 30, 50 or 80 mgl^{-1} NO_2. Similar to the results of prolonged nitrite exposure, acute exposure to high nitrite concentration induced slight changes in tissue composition. However, a clear trend of decreasing liver glycogen was observed in treated fish. In addition, muscle lactate content in fish exposed to 50 and 80 mgl^{-1} NO_2-N showed a significant decline as compared with the values of control fish. Liver protein and lactate, and brain lactate were not affected.

6.4.3. Changes in branchial Na^+-K^+-ATPase activity, hepatic enzyme activities, and liver adenine nucleotide levels in response to acute nitrite exposure

Branchial Na^+-K^+-ATPase activity in nitrite-treated fish was significantly lower than that of control fish (Table 6.6). Significant decreases were also found in liver glycogen phosphorylase a, glutamate-oxaloacetate transaminase and glutamate dehydrogenase activities in treated fish as compared with control fish. There was no significant difference in activities of lactate dehydrogenase, isocitrate dehydrogenase, zglucose-6-phosphatase, glutamate-pyruvate transaminase and glucose-6-phosphate dehydrogenase between control and treated fish (Table 6.6).

Changes in contents of liver ATP, ADP and AMP of Lates calcarifer after 96 hour exposure to 50 mgl^{-1} NO_2-N are shown in Table

6.7. We can see that ATP, ADP and AMP levels were all lower in treated fish when compared with control fish, though the decrease was significant only in ADP and AMP. Calculation showed that the adenylate energy charge was not significantly different between treated and control fish.

6.4.4. Changes in ammonia excretion rate in response to acute nitrite exposure

Figure 6.1 records the changes in ammonia content of the water into which control and treated fish were transferred respectively. Apparently, the ammonia content of the water into which treated fish were transferred was consistently higher than that of the water holding the control fish at all time intervals. The average rates of ammonia excretion for control and treated fish were 6.04 $\pm$ 0.63 and 16.37 $\pm$ 0.69 $\mu g\ NH_3\text{-}N\ g^{-1}\ h^{-1}$ respectively. Therefore, nitrite exposure can trigger an increase in production and excretion of ammonia in fish.

6.4.5. Changes in blood NADH-methemoglobin reductase activity in response to acute and prolonged nitrite exposure.

Lates calcarifer was found to possess the NADH-methemoglobin reductase enzyme system. However, the activity of this enyzme was not affected by acute or prolonged exposure to nitrite (Table 6.8).

Table 6.1. Changes in serum ion concentrations in *Lates calcarifer* after prolonged (8-day) exposure to 10 mgl^{-1} NO_2-N

Ion (mM)	Control (n = 13)	Treated (n = 10)
Cl^-	121.38 ± 3.20	127.80 ± 1.62
Na^+	198.15 ± 3.70	218.55 ± 6.58*
K^+	1.66 ± 0.14	2.36 ± 0.20*
Ca^{2+}	1.51 ± 0.06	1.59 ± 0.13

Values are presented as mean ± SEM

*Null hypothesis of no difference between control and treated groups was rejected at $p < 0.05$ (Student's *t*-test)

Table 6.2. Changes in serum metabolite concentrations in *Lates calcarifer* after prolonged (8-day) exposure to 10 mgl^{-1} NO_2-N

	Control (n = 13)	Treated (n = 10)
Glucose (mg 100 ml^{-1})	40.39 ± 4.71	41.13 ± 6.03
Lactate (mM)	1.56 ± 0.23	2.57 ± 0.48
Protein (g 100 ml^{-1})	5.16 ± 0.25	4.79 ± 0.45
Lipid (g 100 ml^{-1})	0.15 ± 0.01	0.10 ± 0.01*
Cholesterol (g 100 ml^{-1})	0.26 ± 0.02	0.24 ± 0.02
d-Amino acid (mg 100 ml^{-1})	69.00 ± 6.40	90.20 ± 8.56
Ammonia (µg NH_3-N ml^{-1})	5.81 ± 0.38	8.52 ± 0.79*
Urea (mM)	1.29 ± 0.11	1.57 ± 0.15

Values are presented as mean ± SEM

*Null hypothesis of no difference between control and treated groups was rejected at $p < 0.05$ (Student's t-test)

Table 6.3. Changes in tissue composition of *Lates calcarifer* after prolonged (8-day) exposure to 10 mgl^{-1} NO_2-N

	Control (n = 13)	Treated (n = 10)
Hepatosomatic index (% body wt)	0.97 ± 0.09	1.37 ± 0.21
Liver water (% wet wt)	51.14 ± 3.98	55.01 ± 4.48
Liver lipid (% dry wt)	63.04 ± 5.14	54.17 ± 7.91
Liver glycogen (mg g^{-1})	14.45 ± 2.11	17.53 ± 2.38
Liver protein (g 100 g^{-1})	8.08 ± 0.81	7.90 ± 0.80
Liver lactate (μmol g^{-1})	3.94 ± 0.40	4.32 ± 0.35
Muscle water (% wet wt)	77.46 ± 0.29	77.75 ± 0.52
Muscle lipid (% dry wt)	2.66 ± 0.55	3.51 ± 0.82
Muscle lactate (μmol g^{-1})	41.74 ± 5.02	42.18 ± 5.83
Brain lactate (μmol g^{-1})	52.40 ± 3.42	50.62 ± 3.81

Values are presented as mean ± SEM

Table 6.4. Changes in serum metabolite concentrations in Lates calcarifer after acute (96-hour) exposure to various nitrite concentrations

	Control (n = 6)	30 mgl^{-1} NO_2-N (n = 6)	50 mgl^{-1} NO_2-N (n = 6)	80 mgl^{-1} NO_2-N (n = 5)
Glucose ($mg\ 100\ ml^{-1}$)	25.53 ± 2.88^{a}	21.60 ± 3.87^{a}	16.35 ± 3.61^{a}	16.54 ± 2.76^{a}
Lactate (mM)	0.46 ± 0.16^{a}	1.10 ± 0.45^{a}	1.41 ± 0.22^{a}	5.03 ± 0.69^{b}
Protein ($g\ 100\ ml^{-1}$)	6.02 ± 0.10^{a}	5.96 ± 0.28^{a}	6.20 ± 0.28^{a}	5.06 ± 0.35^{b}
Ammonia ($\mu g\ NH_3$-N ml^{-1})	5.87 ± 1.08^{a}	n.d.	11.92 ± 0.98^{b}	13.71 ± 0.80^{b}
Urea (mM)	1.07 ± 0.12^{a}	n.d.	1.36 ± 0.10^{a}	1.88 ± 0.26^{b}

Values are presented as mean $\pm$ SEM. n.d.: value not determined

Values with different alphabets are significantly different at $p = 0.05$ (One Way Analysis Of Variance followed by Duncan's Multiple Range Test).

Table 6.5. Changes in tissue composition of Lates calcarifer after acute (96-hour) exposure to various nitrite concentrations

	Control (n = 6)	30 mgl^{-1} NO_2-N (n = 6)	50 mgl^{-1} NO_2-N (n = 6)	80 mgl^{-1} NO_2-N (n = 5)
Liver glycogen ($mg\ g^{-1}$)	18.31 ± 6.71^{a}	7.68 ± 1.42^{b}	6.96 ± 2.22^{b}	3.89 ± 0.96^{b}
Liver protein ($g\ 100\ g^{-1}$)	8.10 ± 0.35^{ab}	8.36 ± 0.41^{b}	8.31 ± 0.34^{a}	8.66 ± 0.23^{b}
Liver lactate ($\mu mol\ g^{-1}$)	3.56 ± 0.29^{a}	2.75 ± 0.54^{a}	2.68 ± 0.35^{a}	2.95 ± 0.18^{a}
Muscle lactate ($\mu mol\ g^{-1}$)	32.82 ± 1.46^{c}	29.66 ± 1.74^{bc}	21.53 ± 3.09^{a}	25.18 ± 1.58^{ab}
Brain lactate ($\mu mol\ g^{-1}$)	50.03 ± 3.77^{a}	42.55 ± 2.51^{a}	48.08 ± 2.20^{a}	47.96 ± 5.44^{a}

Values are presented as mean ± SEM.

Values with different alphabets are significantly different at $p = 0.05$ (One Way Analysis Of Variance followed by Duncan's Multiple Range Test).

Table 6.6. Effects of acute (96-hour) exposure to 50 mgl^{-1} NO_2-N on gill and liver enzymatic activities of Lates calcarifer

	Control (n = 6)	Treated (n = 6)
Gill:		
Na^+-K^+-ATPase (umol Pi mg protein^{-1} h^{-1})	8.34 ± 0.58	4.70 ± 0.37*
Liver:		
Phosphorylase a (umol G1P g protein^{-1} min^{-1})	100.13 ± 3.06	68.68 ± 5.86*
LDH (umol g protein^{-1} min^{-1})	84.81 ± 7.96	84.96 ± 6.20
ICDH (umole g protein^{-1} min^{-1})	211.02 ± 13.23	239.28 ± 14.53
G6Pase (umole Pi g protein^{-1} min^{-1})	23.25 ± 3.60	18.73 ± 3.68
GPT (units mg protein^{-1})	97.19 ± 6.82	108.36 ± 7.26
GOT (units mg protein^{-1})	46.97 ± 1.67	34.21 ± 1.96*
G6PDH (umole g protein^{-1} min^{-1})	148.24 ± 4.78	151.06 ± 5.32
GlDH (umole g protein^{-1} min^{-1})	11.91 ± 0.68	9.90 ± 0.54*

Values are presented as mean ± SEM

*Null hypothesis of no difference between control and treated groups was rejected at $p < 0.05$ (Student's t-test)

Table 6.7. Changes in concentrations of liver adenosine nucleotides of *Lates calcarifer* after acute (96-hour) exposure to 50 mgl^{-1} NO_2-N

	Control (n = 6)	Treated (n = 6)
Adenosine triphosphate (ATP) ($\mu mol\ g^{-1}$)	3.04 ± 0.55	1.90 ± 0.55
Adenosine diphosphate (ADP) ($\mu mol\ g^{-1}$)	1.83 ± 0.26	0.82 ± 0.17*
Adenosine monophosphate (AMP) ($\mu mol\ g^{-1}$)	1.14 ± 0.25	0.34 ± 0.07*
Adenylate energy charge (AEC)	0.55 ± 0.06	0.74 ± 0.03

Values are presented as mean ± SEM

*Null hypothesis of no difference between control and treated groups was rejected at $p < 0.05$ (Student's *t*-test)

Table 6.8. Changes in NADH-methemoglobin reductase activity of *Lates calcarifer* in response to acute and prolonged nitrite exposure

	Control (n = 4)	Treated (n = 4)
Acute exposure (96 hours, 50 mgl^{-1} NO_2-N)		
NADH-methemoglobin reductase ($\mu mol\ min^{-1}\ ml\ cell^{-1}$)	0.54 ± 0.11	0.40 ± 0.05
Prolonged exposure (8 days, 10 mgl^{-1} NO_2-N)		
NADH-methemoglobin reductase ($\mu mol\ min^{-1}\ ml\ cell^{-1}$)	0.44 ± 0.03	0.41 ± 0.02

Values are presented as mean ± SEM

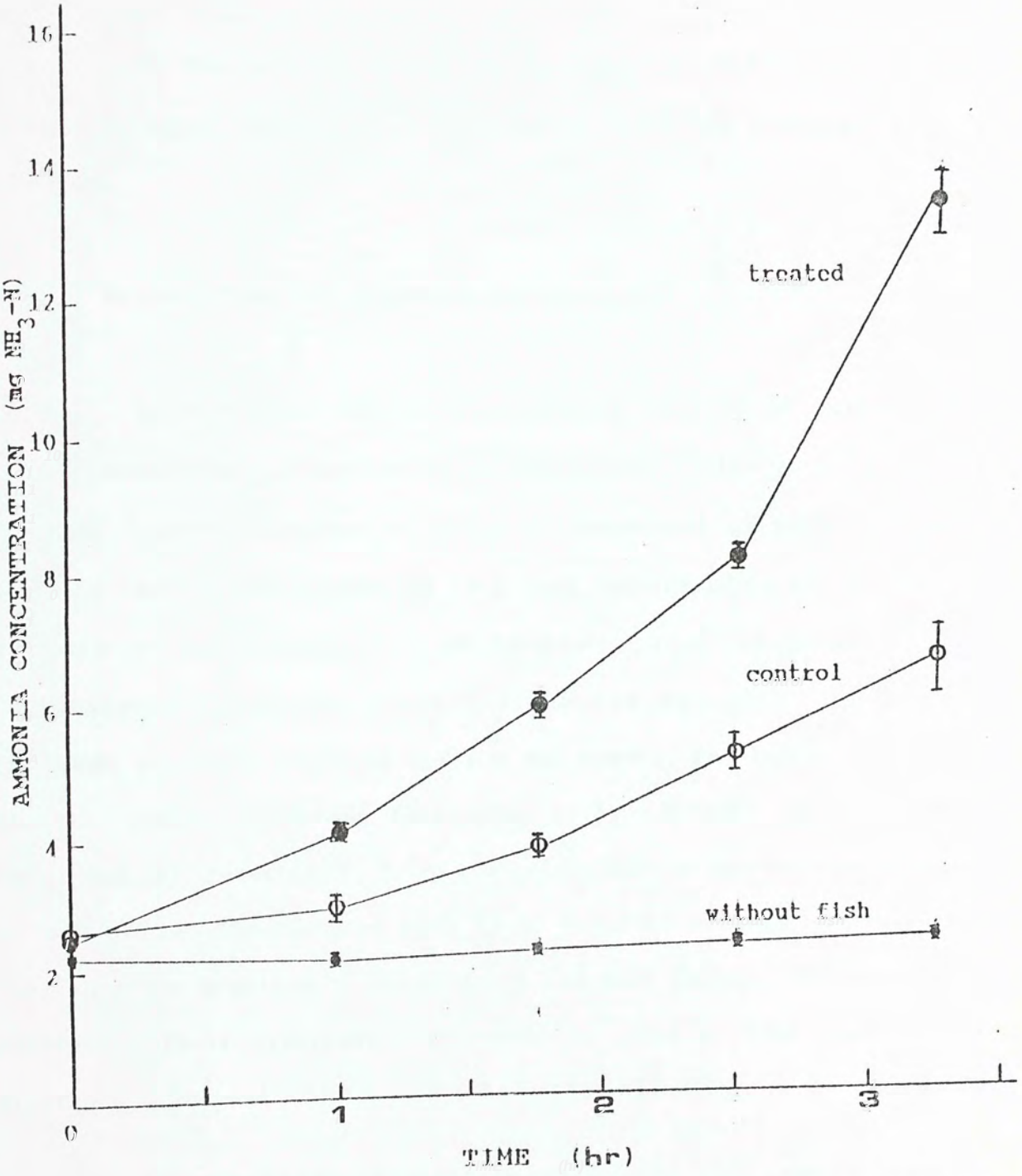

Figure 6.1. Changes in the ammonia concentration of the water (Vol.=20-l) into which control (O) or treated (●) fish were transferred. (•) without fish.

6.5. DISCUSSION

In the present study, we attempt to study the metabolic changes in Lates calcarifer in response to acute and prolonged nitrite exposure.

6.5.1. Serum sodium and potassium accumulation

Teleost fish exhibit a remarkable ability to maintain the ionic composition and osmolarity of their body fluids at a relatively constant levels regardless of their environmental salinity. Studies over the last 50 years have led to a firm understanding of the general pattern of osmoregulatory mechanisms, and the head region, particularly the gills, clearly plays the key role in the salt transport processes required for ion and osmotic regulation (Doyle and Gorecki, 1961; Maetz and Bornancin, 1975; Foskett et al., 1983). The blood of seawater fish or seawater-adapted euryhaline fish is markedly hypotonic compared with their external milieux and therefore they face the problems of dehydration and salt influx. To be able to survive in their hypertonic environments, seawater fish continuously drink sea water and excrete salts by extrarenal means.

The so-called chloride cells in the gill epithelium of marine or euryhaline fish have long been shown to be responsible for the active excretion of salts into hypertonic environments (Doyle and Gorecki, 1976; Utida et al., 1971). Branchial Na^+-K^+-ATPase which is

localized on the basolateral surface of the chloride cells has been repeatedly shown to be involved in salinity adaptation in many euryhaline species. Studies concerning the role of branchial Na^+-K^+-ATPase generally held that this enzyme is involved in the active extrusion of NaCl from the gill in fish adapted to sea water (Epstein et al., 1967; Sargent et al., 1975; Epstein et al., 1980). Therefore, in many euryhaline fish species, Na^+-K^+-ATPase activity is markedly elevated upon adaptation to high salinities (Epstein et al., 1967; Utida et al., 1971; Sargent and Thomson, 1974; Ho and Chan, 1980; Woo and Tong, 1982).

In the present study, exposure of Lates calcarifer to 10 mgl^{-1} NO_2-N for 8 days resulted in increased serum Na^+ and K^+ concentrations. However, this was not accompanied by decrease in hematocrit or tissue water content. Therefore, the elevated serum Na^+ and K^+ concentrations were certainly not due to body dehydration but some defects in the ion extrusion mechanisms.

In order to further investigate the impact of nitrite on the osmoregulation of Lates calcarifer, a separate batch of fish was subject to 96 hour exposure of 50 mgl^{-1} NO_2-N and the branchial Na^+-K^+-ATPase activity was assayed. It was found that the branchial Na^+-K^+-ATPase activity in nitrite-treated fish was markedly lower than that of control fish. This finding indicates that nitrite can offset the osmoregulation of fish by markedly inhibiting the activity of one of the important branchial transport enzyme Na^+-K^+-ATPase. Since

branchial Na^+-K^+-ATPase is responsible for the active extrusion of Na and Cl across the gills, inhibition of the activity of this enzyme should result in an accumulation of serum chloride in addition to serum sodium. In fact, as shown in Table 6.1, the serum chloride level tends to be higher, though insignificantly, in treated fish as compared with control fish. Lack of significance may be due to the small sample size or increased excretion of chloride ion through other pathways (e.g. renal) or decreased absorption of chloride ion through the gut. Serum calcium level was not elevated, presumably due to the relatively small ionic gradient that exists between sea water and serum. Also, there is no evidence that the gill participates in the calcium excretion. Therefore, gill damage or branchial transport enzyme inhibition may not interfere calcium level in the fish blood.

Inhibitory effects of nitrite on branchial transport enzyme has not been reported for other marine or euryhaline fish. However, for freshwater fish, nitrite has been shown to markedly inhibit the activity of branchial carbonic anhydrase *in vitro* in rainbow trout (Gaino *et al.*, 1984). Carbonic anhydrase plays an important role in the chloride absorption of freshwater fish. It catalyzes the production of bicarbonate which is believed to exchange for chloride in the external environment (Maetz and Garcia-Romeu, 1964). Therefore, no matter in freshwater or seawater fish, nitrite definitely confers osmoregulatory failure on them.

6.5.2. Glycogen depletion and lactate accumulation

An 8 day immersion in 10 mgl^{-1} NO_2-N did not elicit marked metabolic changes in Lates calcarifer. Glycogen depletion and lactate accumulation appear to be the most important phenomena which occurred when fish are subject to hypoxia or anoxia (e.g. Heath and Pritchard, 1965; Walker and Johanson, 1977; Van der Thillart et al., 1980; Woo and Wu, 1984; Yu and Woo, 1987). These two responses, however, were not observed in Lates subject to prolonged nitrite exposure. However, accumulation of serum lactate was evident when Lates were exposed for 96 hours to 80 mgl^{-1} NO_2-N at which more than 70 % of the fish hemoglobin was oxidized to methemoglobin. The lactate in serum may be chanelled from other organs where anaerobiosis occurred or produced locally by the red blood cells. It has been argued that the brain is the most vulnerable organ to oxygen deprivation (Robin et al., 1964) while white muscle is capable of prolonged anaerobiosis. In this experiment, we did not observe an increase in brain lactate content in fish exposed to any one of the test concentrations. Lack of increase in liver lactate content was also observed in treated fish. Absence of elevation in lactate content in both brain and liver indicated that these two organs did not suffer from oxygen deprivation and anaerobiosis even when less than 30 % of the hemoglobin was left for oxygen transport. At extreme high nitrite concentration (eg. 80 mgl^{-1} NO_2-N), physiological adjustments should have been made so that adequate oxygen was provided to those essential organs including brain

and liver. Capillary dilatation may be one physiological adjustment that increases the perfusion of organs. Capillary dilatation in response to hypoxia has been demonstrated in brain of fish (Scheich et al., 1972). L. calcarifer did not accumulate lactate in the white muscle during nitrite exposure. Partly because of the sluggish habit, L. calcarifer showed very low activity levels during the experimental period. It is well known that at very low activity levels white muscles are not used in locomotion and therefore consume little energy. The apparent absence of activation of anaerobic metabolism in white muscle during nitrite exposure indicated that nitrite posed no significant increase in metabolic load on the muscle. Instead, the fish subject to nitrite exposure became even more sluggish when compared with control fish. One interesting finding was that there was a tendency of decreasing muscle lactate content as exposure concentration of nitrite increased and the decrease became significant in fish exposed to 50 and 80 mgl^{-1} NO_2-N. It is well known that lactate is a dead end in metabolism. Lactate diffuses out of active skeletal muscle into the blood and are carried out to the liver where lactate is converted into glucose by gluconeogenesis. Hepatic gluconeogenesis from lactate has been demonstrated in some teleosts (Phillips and Hird, 1977; Mosse, 1980; Renaud and Moon, 1980). Glucose then enters the blood and is taken up by skeletal muscle or other tissues. At severe nitrite intoxication, there was a great demand for metabolic energy to resist the stress and therefore the lactate in muscle was probably transported to the liver where it was

converted to the metabolic fuel, glucose.

Lates calcarifer was shown to mobilize liver glycogen during acute nitrite exposure. Glycogen mobilization in liver has been demonstrated in the red grouper and the black sea bream during hypoxic exposure (Woo and Wu, 1984). Increased glycogen breakdown in liver should increase the serum glucose. However, this was not the case in the present experiments. The serum glucose concentrations in treated fish were not significantly different from that of control fish. This could probably be attributed to an increased uptake of glucose from the blood stream by various tissues in order to provide fuel for the extra metabolic energy requirement during the experimental period. In addition, increased consumption of glucose to generate more energy by red blood cells may also account for the unchanged level of serum glucose in treated fish. The elevated serum lactate suggest an enhanced anaerobiosis in the erythrocytes of L. calcarifer, at least, during acute exposure to 80 mgl^{-1} NO_2-N. Anaerobic metabolism was activated in red blood cells because of oxygen deprivation which resulted from the high methemoglobin concentration. Besides, a large amount of ATP was urgently required by the methemoglobin reductase enzyme system to reduce methemoglobin back to hemoglobin. Under enhanced nitrite intoxication, formation of lactate through anaerobic pathway can produce considerable amount of energy in a short period of time.

6.5.3. Serum lipid and protein depletion

Prolonged (8 day) exposure of the fish to 10 mgl^{-1} NO_2-N caused no depletion of lipid in liver and muscle. However, a depletion was observed in serum lipid content. Lipid is a major metabolic fuel in animals. Considerable amount of the serum lipid may be broken down to fatty acids and glycerol of which energy is readily generated to meet the energy requirement of the red blood cells or other tissues. On the other hand, the serum cholesterol content remained unchanged during prolonged nitrite exposure.

Generally speaking, nitrite exposure did not elicit pronounced effect on the protein content of the fish. Liver protein remained unchanged after prolonged or acute nitrite exposure although protein has been reported to be the major energy source in fish (Nagai and Ikeda, 1971). Serum protein was significantly decreased only in fish exposed to 80 mgl^{-1} NO_2-N. A decline in serum total protein levels has also been recorded during the acute and chronic exposure of Clarias lazera to nitrite (Hilmy et al., 1987). The decline in serum protein may be a generalized stress response since a great variety of stressors including toxicants also tend to diminish the total blood protein (Selye, 1950).

6.5.4. Serum ammonia and urea accumulation

Prolonged and acute nitrite exposure caused an elevated serum

ammonia in Lates calcarifer. The elevated serum ammonia suggests an increase in the excretion of ammonia in treated fish. The experiment conducted to compare the rate of ammonia excretion of nitrite-treated fish with that of control fish supported this speculation. The average ammonia excretion rate of treated fish was about 3 fold of that of control fish.

Teleostean fish excrete the major part of their nitrogenous waste in the form of ammonia. Ammonia accounts for 55-80 % of the total nitrogen excreted (Wood, 1958; Fromm, 1963). Relatively little information is available about the pathways of nitrogen metabolism in fish. Most fish are carnivorous with a high dietary protein intake. A relatively large part of their energy is derived from catabolism of proteins and therefore protein is considered as the main source of excreted nitrogen in fish (Walton and Cowey, 1977). Several metabolic routes have been proposed as major mechanisms for amino acid deamination in fish tissues, but none of them can be considered as established, because no conclusive evidence has been presented about their existence, maximal capacity and quantitative operation in vivo (Waarde, 1981). One of these mechanisms is the transdeamination scheme (Braunstein, 1957; cited in Waarde, 1981), in which amino acids transaminate with α-ketoglutarate to form glutamate, which is subsequently deaminated in the mitochondrial matrix by the endergonic glutamate dehydrogenase reaction. Another route is the cytosolic, exergonic purine nucleotide cycle, proposed by Braunstein (1957)

(cited in Waarde, 1981) and Lowenstein (1972), in which AMP deaminase catalyzes the deamination of AMP to produce ammonia. Furthermore, specialized deamination pathways may exist for the catabolism of aspartate, cysteine and histidine (Salvatore et al., 1965), although their quantitative importance in ammonia production must be questioned (Pequin and Serfaty, 1963).

The increased production of ammonia in treated fish may result from an increase in protein degradation and amino acid deamination which can generate more energy for the resistance of nitrite toxicity. However, a significant reduction in serum or liver protein content was not evident in treated fish except those exposed to 80 mgl^{-1} NO_2-N. Absence of measurable reduction in liver protein strongly suggests the possibility that the purine nucleotide cycle is activated in treated fish so that additional amount of ammonia arises from the deamination of AMP. This point will be further elaborated in a subsequent section in combination with the results of liver enzyme activities and adenine nucleotide levels.

It is generally held that fish do not have a complete urea cycle. However, exceptional cases are not uncommon. For example, it has been well established that the lungfish has a full complement of ornithine-urea cycle enzymes in the liver and is shown to be able to synthesize urea under condition of unfed and estivation (Janssens and Cohen, 1967). Nevertheless, until quite recently, there was still strong controversial opinions about the existence of a functional

ornithine-urea cycle in teleosts. Two enzymes of the cycle, carbamylphosphate synthetase and ornithine transcarbamoylase, have been reported to be absent from teleost liver (Brown and Cohen, 1960). Channel catfish has shown to have an incomplete ornithine-urea cycle (Wilson, 1971). However, Huggins et al. (1969) found significant levels of all five enzymes of the urea cycle in several species of teleostean fish, both from freshwater and marine habitats. High activities of all five of the urea cycle enzymes have also been demonstrated in marine teleost Opsanus tau (Read, 1971).

The present study has demonstrated that the serum urea content in treated fish was higher than that of control fish. Arginase, an enzyme of the urea cycle, has been shown to be present in the liver of Lates calcarifer (Lee and Woo, unpublished). However, another urea cycle enzyme, ornithine transcarbamylase could not be detected. Therefore, L. calcarifer are unlikely to possess a complete urea cycle and the urea found in the serum may arise from uricolysis. Fish exposed to nitrite seemed to have an enhanced purine degradation during which AMP is deaminated to IMP by AMP deaminase and ammonia is evolved. Further breakdown of IMP results in the formation of urea. The increased producton of ammonia and urea by purine degradation may account for the elevated serum ammonia and urea level in treated fish.

6.5.5. Changes in hepatic enzyme activities and adenine nucleotide levels

In order to have a clearer understanding of the underlying action of nitrite, the activities of enzymes involved in several major biochemical pathways in liver were determined. Based on the available data, fish exposed 96 hours to 50 mgl^{-1} NO_2-N did not show marked changes in liver metabolic contents. This was the same for hepatic enzyme activities. Significant changes were only observed in activities of phosphorylase, GOT and GlDH. Absence of changes in activities of ICDH, LDH, G6PDH and G6Pase suggests that nitrite seems to have little influence on the several biochemical pathways including TCA cycle, anaerobiosis, pentose phosphate pathway and gluconeogenesis.

It is well known that phosphorylase exists in two interconvertible forms: an active phosphorylase a and a usually inactive phosphorylase b. Only the active form can catalyze the glycogenolytic reaction. Conversion of the inactive form to active form involves a sequence of reactions which are catalyzed by a series of enzymes including adenylate cyclase, protein kinase and phosphorylase kinase. As shown in Table 6.5, the treated fish tended to exhibit a lower liver glycogen content when compared with the control fish. However, the treated fish showed a decreased activity of liver glycogen phosphorylase a. These two observations seemed to be contradictory. The possible reason was that during the initial

period of nitrite exposure, glycogen breakdown was really enhanced in treated fish in order to provide more energy for the resistance of nitrite toxicity. However, after a certain period of nitrite intoxication, one or more of those reactions that convert the phosphorylase from inactive form to active form might be blocked by nitrite so that a lower phosphorylase a activity resulted in the treated fish. In addition, nitrite may exert its inhibitory effect on glycogen phosphorylase a directly.

Glutamate-oxaloacetate transaminase (GOT) and glutamte-pyruvate transaminase (GPT) catalyze the transamination of aspartate and alanine respectively with α-ketoglutarate to form glutamate. Since the liver glutamate dehydrogenase activity was significantly lowered in treated fish, the glutamate concentration may rise to a level which can inhibit the activities of GOT and GPT which catalyze the formation of glutamate. Treated fish showed a significant decrease in liver GOT activity. Absence of change in GPT activity may due to the fact that GPT is responsible for the transamination of alanine which serves as an intermediate product for the transamination of other -amino acids (Stryer, 1975).

Glutamate dehydrogenase is generally held to play a central role in amino acid deamination and nitrogen excretion by animals (Walton and Cowey, 1977). Although, thermodynamically, the glutamate dehydrogenase reaction favours reductive amination of α-ketoglutarate rather than oxidative deamination of glutamate, an equilibrium is

considered to arise *in vivo* which due to factors such as relative levels of nucleotides and removal of reaction products favours glutamate oxidation (Walton and Cowey, 1977). Glutamate dehydrogenase catalyzes the following reaction:

$$\text{glutamate} + \text{NAD(P)}^+ + H_2O \longleftrightarrow \alpha\text{-ketoglutarate} + NH_4^+ + \text{NAD(P)H} + H^+$$

An apparent increase in ammonia production was observed in treated fish. If ammonia only comes from the glutamate dehydrogenase reaction, the increased serum ammonia level in treated fish may imply that the activity of glutamate dehydrogenase in the liver of treated fish should be higher than that of control fish. However, this was not the case in the present study. The activity of GLDH in treated fish was significantly lowered. This result indirectly confirms the possibility that the additional amount of ammonia found in treated fish arises from the purine nucleotide cycle in which AMP deaminase degrades AMP to produce inosine monophosphate (IMP) and ammonia. Nevertheless, from the present available data, it is difficult to draw a conclusion about the relative importance of the purine nucleotide cycle and glutamate dehydrogenase reaction in ammonia production of the fish. In rat tissues AMP deaminase activity appears to be inversely proportional to those of glutamate dehydrogenase (Lowenstein, 1972), and this is also true for trout (Walton and Cowey, 1977) and goldfish (Waarde, 1980). A high activity of the former is accompanied by a low activity of the latter, and vice versa. The

present experiments have not measured the AMP deaminase activity in both control and treated fish. However, the increased degradation of AMP suggests an enhanced activity of AMP deaminase in treated fish. The enhanced activity of AMP deaminase may have an inhibitory effect on those of glutamate dehydrogenase so that its activity becomes significantly lowered in treated fish.

The adenine nucleotide pools have been proposed as a system that responds rapidly and sensitively to stress to indicate adverse conditions before they are irreversible in the organism (Giesy, 1988). Stress including hypoxia, anoxia and heat have been widely shown to affect the levels of adenine nucleotides in fish (Van den Thillart et al., 1976; Van den Thillart et al., 1980; Giesy, 1988). Therefore, experiments were carried out to measure the liver ATP, ADP and AMP levels in order to investigate whether nitrite has any adverse effects on the energy pool of the treated fish. As shown in Table 6.7, a significant decrease in liver ADP and AMP concentrations could be observed in treated fish. Calculation showed that the adenylate energy charge (AEC) which was defined by Atkinson(1968) as (ATP + 1/2 ADP) / (ATP + ADP + AMP) are not significantly different between treated and control fish.

Recent biomedical research has shown that various forms of stress such as disease, injury or exposure to pollutants act in a

similar manner by eliciting physiological defense responses (Selye, 1976). Because of the metabolic activity associated with these defenses, increased energy utilization occurs when an organism is subject to a stressor. Therefore, it is within our expectation that a decrease occurs in liver ATP which is the primary source of biochemical energy during nitrite immersion period. The decreased availability of ATP together with an increased demand for energy to resist the stressor result in an inadequate energy supply to carry out normal cellular function and maintain homeostasis. ATP is the organisms' "energy currency" and its concentration can directly increase the activity of some enzymes, while decreasing the activity of others. For example, at greater ATP concentrations, metabolism is shifted toward gluconeogenesis due to decreased activity of phosphofructokinase. When ATP concentrations are small, metabolism is shifted toward glycolytic degradation of glucose due to AMP inhibition of fructose diphosphatase (Giesy, 1988).

An interesting phenomenon in treated fish could be observed with regard to liver ADP and AMP concentrations: liver ADP and AMP concentrations decreased as ATP concentration decreased. If ATP is discharged directly to ADP and AMP, decrease in ATP level should be accompanied by a concomitant increase in ADP and AMP levels. However, we could see that the total sum of liver ATP, ADP and AMP decreased; a finding which indicated that adenine nucleotides disappeared in nitrite-treated fish. With regard to the standard energy charge model, Giesy (1988) stated that during short-term stress, the energy

associated with ATP would be utilized so that the ATP concentration would decrease while the ADP, and subsequently AMP, concentrations increase. Therefore, under short-term stress, one would expect to observe a negative correlation between both ADP and AMP concentrations and the concentration of ATP. Because the relative concentrations of adenylates are thought to be closely regulated (Atkinson, 1968), one would expect that under long-term stress the concentration of AMP would be reduced by degradation in order to bring the adenylate energy charge back to homeostatic values, while the total concentration of adenylates is reduced. Decrease in total sum of ATP, ADP and AMP levels has also been reported in goldfish exposed to anoxia (Van den Thillart et al., 1976).

Adenylate energy charge has been suggested as a measure of the metabolic energy available to an organism from the adenylate pool (Hava and Waiwood, 1983) and theoretically it can range between 0 (all AMP) and 1.0 (all ATP). Under optimal conditions the AEC of actively metabolizing cells and tissues or organisms from bacteria to mammals ranges between 0.75 and 0.99 (Chapman et al., 1971; Atkinson, 1977). While lower values in the range of 0.55 and 0.75 indicate stress conditions and below 0.50 death is normally imminent (Chapman et al., 1971; Montague and Dawes, 1974; Beis and Newsholme, 1975; Atkinson, 1977; Holmsen and Robkin, 1977). Energy charge is highly regulated (Atkinson, 1968) and seems to change only when organisms are moved out of their region of homeostasis. For example, in goldfish exposed to

hours to anoxia, the energy charge of liver decreased from 0.60 to 0.32 (Van den Thillart et al, 1980). Adenylate energy charge is kinetically important in the enzyme regulation of catabolic, amphibolic and anabolic sequences (Atkinson, 1968; Ebberink et al, 1976). Greater values cause increased anabolism, while lower ratios induce increased catabolism.

The present study found that the adenylate energy charge of treated and control fish were 0.65 and 0.74 respectively. Both values are relatively lower than those of mammals (Kamiyama et al., 1976) but quite comparable to those reported for goldfish (Van den Thillart et al., 1980), trout (Gras et al., 1967; cited in Van den Thillart et al., 1980), striped catfish (Yip, S. W., personal communication) and Chinese mudskipper (Yip, S. W., personal communication). The two values obtained fall in the range of 0.55 and 0.75 which indicates stress conditions. Since control fish were unfed during the experimental period, starvation might pose considerable stress in the fish. Stabilization of adenylate energy charge in liver tissue of treated fish can partly be achieved by an enhanced production of ATP or an increased conversion of AMP into IMP. The concentration of liver ATP in treated fish, however, was found to be unchanged. Therefore, most likely treated fish decreased their liver AMP concentrations by converting it into IMP in order to maintain their adenylate energy charge at a relatively constant value. IMP probably is the substance into which all adenosine-compounds are converted.

(Murray and Jones, 1957; Saito et al., 1959) but also after severe exercise (Jones and Murray, 1960; Fraser et al., 1966). The accumulation of IMP results from action of the enzyme AMP deaminase, which catalyses the reaction AMP + H_2O ———› IMP + NH_3. This enzyme has been described in carp, mackerel and cod muscle (Dingle and Hines, 1967; Hidaka and Saito, 1960) and proved to be highly active (± 400 µmol min^{-1} g^{-1}). The physiological significance of IMP-accumulation was described by Tornheim and Lowenstein (1972) in rat muscle. They found that the two antagonistic enzymes, AMP deaminase and AMP synthetase, were regulated by the energy charge. At high energy charge AMP was produced and at low energy charge AMP was converted into IMP. According to the reaction mentioned above, we can see than ammonia is also produced when AMP is degraded to IMP. It is clear that increased degradation of AMP to IMP should lead to concomitant increase in ammonia production. Probably this could also explain the increase in ammonia production in fish exposed to nitrite.

6.5.6. Changes in NADH-methemoglobin reductase activity

Methemoglobin is continuously formed in normal erythrocytes. Small amounts of methemoglobin (1 %) occur in mammalian red blood cells (Beutler, 1968). In poikilotherms, erythrocytic methemoglobin contents have been reported to be 2.9 % in rainbow trout (Cameron, 1971), 2.2 % in channel catfish (Huey et al., 1980), and 5.7 % in Rana catesbiana tadpoles (Huey and Beitinger, 1980a).

Humans possess a methemoglobin reductase system capable of converting methemoglobin to hemoglobin (Jaffe, 1964). Scott et al. (1965) have demonstrated that in man reduction is accomplished principally by the enzyme NADH-methemoglobin reductase (Diaphorase I), although reduced glutathione, ascorbic acid, and NADPH-methemoglobin reductase appear to reduce methemoglobin to a lesser extent. Erythrocytic methemoglobin reductase activity has been studied in several common mammalian species (Agar and Harley, 1972).

Huey et al. (1980) found that channel catfish recovered from extreme methemoglobinemia (80 % of total hemoglobin) within 24 hours when transferred to nitrite-free water. This suggests that a methemoglobin reductase system similar to that active in mammalian erythrocytes is operating in catfish. This hypothesis was first proposed by Cameron (1971) and supported experimentally by Huey and Beitinger (1982). Freeman et al. (1983) further confirmed the presence of NADH-methemoglobin reductase system in 10 of the 13 freshwater fish species tested. Later, Scott and Harrington (1985) determined the reductase activity of rainbow trout and coho salmon and compared with that of human. However, no similar studies have been directed to study the reductase enzyme system of marine or euryhaline fish.

In the present experiment, Lates calcarifer was found to possess the NADH-methemoglobin reductase system and the activity measured at 23 ^{o}C was equal to 0.44 μmol min^{-1} ml^{-1} cell. The only

data available in the literature on the activities of NADH-methemoglobin reductase were reported by Scott and Harrington (1985). They found that the NADH methemoglobin reductase activity of both the rainbow trout (0.036 μmol min^{-1} ml^{-1} cell) and coho salmon (0.059 μmol min^{-1} ml^{-1} cell) was higher than that of human (0.031 μmol min^{-1} ml cell) at 15°C. Since the available data on the activities of NADH-methemoglobin reductase were obtained at 15 °C, it was quite inappropriate to compare the results obtained in the present study with them. However, the NADH-methemoglobin reductase activity of Lates calcarifer at 23°C was nearly ten fold of that of rainbow trout, coho salmon and human measured at 15 °C. We can infer that L. calcarifer is equipped with a relatively high activity of NADH-methemoglobin reductase. This speculation is in line with the finding that L. calcarifer has a low basal methemoglobin level (See Section 5.5.1.2). High reductase activity would be a distinct physiological advantage because fish hemoglobins undergo autooxidation more readily than mammalian hemoglobins (Borgese et al., 1977; Harrington, 1982; 1983).

Blood samples of fish differed from those of human in that they required the presence of a detergent, such as Triton X-100, for solubilization of the methemoglobin reductase system (Scott and Harrington, 1985). To assay the reductase activity of Lates calcarifer, Triton X-100 should be added in the hemolysate, otherwise little or no enzymatic activity was observed. This requirement for a

detergent is similar to that observed by Board et al. (1977) for reductase activity in nucleated erythrocytes of reptiles and birds. Freeman et al. (1983) added saponin to the hemolysate preparations prior to carrying out enzyme essays in a series of phylogenetically diverse fish species.

The NADH-methemoglobin reductase activity of Lates calcarifer was not affected by acute or prolonged nitrite exposure. One possibility is that the fish simply did not elevate their reductase activity under the present combination of nitrite dosage and exposure duration, a higher nitrite concentration or a longer exposure period may be required to induce the activity. Other possible reason is that the gene controlling the production of NADH-methemoglobin reductase is noninducible, no matter how high the substrate (methemoglobin) concentration is. This may be a physiological disadvantage for L. calcarifer to tackle the problems of methemoglobinemia caused by nitrite.

6.5.7. Conclusion

In contrast to the pronounced effects on the hematology, nitrite did not elicit marked changes in Lates calcarifer. Based on the available data, it can be concluded that hypoxic symptoms were certainly not elicited in fish exposed to nitrite concentration as low as 10 mgl^{-1} NO_2-N. However, some hypoxic symptoms including lactate accumulation, glycogen depletion and adenine nucleotides depletion

were evident in fish exposed to high concentrations of nitrite, at least at concentrations of 50 and 80 mgl^{-1} NO_2-N. On the other hand, nitrite exposure definitely can induce an increase in ammonia excretion in Lates.

CHAPTER SEVEN GENERAL CONCLUSION

GENERAL CONCLUSION

With the exponential increase in world population, there is a pressing need for the increased production of different types of food. Fish is an important food which is a rich protein source and therefore the demand on fish production, whether from natural capture or fish farming, has become increasingly high. However, due to the global deteriorating water quality arising from pollution and the unmanaged exploitation of fishery resources, fish landing has seen a marked decrease over the recent years. This highlights the greater dependence on intensive fish farming to meet the increasing demand for fish.

In the development of intensive fish farming, there is an urgent need to develop effective re-circulating closed systems because considerable difficulty is involved in obtaining good quality water for intensive culture in many parts of the world. Various types of closed re-circulating aquaculture systems for fish production have been developed in recent years (Liao and Mayo, 1972, 1974; Lewis and Buynak, 1976; Nagel, 1977, 1980; Muir, 1982; Provenzano and Winfield, 1987; Woo et al., 1988). In Hong Kong and other parts of the world, intensive culture of freshwater fish by closed recirculating systems has been attempted with some success. However, in many areas including Hong Kong, the culture of marine food fish is far from ideal in that fish are cultured in float cages around coastal waters (Woo et al, 1988). These culture systems are constantly

exposed to the environment and thus are adversely affected by many environmental conditions, such as red tide and accompanying hypoxia, changes in salinity, pH and temperature, typhoons, disease organisms and various pollutants. It is therefore desirable to develop a re-circulating sea water system for fish production in Hong Kong (Woo et al., 1988).

All re-circulating systems involve the use of a biological filter of some sort (eg. trickling type, submerged type, updraft type, biodiscs, gravel bed, activiated sludge type) to remove the waste products through bacterial action. Two nitrifying bacteria, Nitrosomonas and Nitrobacter flourish on the filters and are responsible for purifying the water. The principal nitrogenous waste, ammonia, is oxidized to nitrite by Nitrosomonas and then subseqently nitrite is oxidized to nitrate by Nitrobacter. Imbalance in the growth of these two genera of bacteria caused by adverse environmental conditions such as high pH and ammonia concentration (Russo, 1985) results in an accumulation of nitrite. Accumulation of nitrite is one of the most critical alterations of water quality in intensive culture systems. Although this may be a shorted-lived phenomenon because of the rapid oxidation of nitrite to nitrate, the highly toxic nature of nitrite to fishes warrants deeper consideration.

In the present study, the 96 hour median lethal concentrations for Lates calcarifer in freshwater, 15 $^{o}/_{oo}$ sea water and 32 $^{o}/_{oo}$ sea water have been established. L. calcarifer is a very

important food fish in Hong Kong. In recent years, aquaculture of this species has developed very rapidly due to the economic feasibility of commercial production of this food fish. Its fast growth rate and remarkable tolerance to fluctuations of some environmental factors such as salinity and dissolved oxygen probably contribute to its popularity in being selected as a cultured species. A pilot experiment to evaluate the effectiveness of a closed biodisc system in purifying sea water for the culture of Lates has been initiated in Hong Kong (Woo et al., 1988). In that study, the biodiscs were able to keep water nitrite concentration below 10 mgl^{-1} NO_2-N at a high stocking density of 7.3 gl^{-1}. Since the LC50 of nitrite for Lates in 32 $^{o}/_{oo}$ is considerably higher than the water nitrite concentrations observed by Woo et al. (1988), intensive culture of this species in closed system should not pose any problem as far as nitrite toxicity is concerned. The LC50 data obtained in the present study also serve as a guideline for the manipulation of water chemistry to prevent nitrite toxicity. For example, it is most feasible to cultivate Lates in 15 $^{o}/_{oo}$ sea water because it has the highest tolerance to nitrite at this salinity.

Most literature concerning nitrite toxicity so far reported mainly dealt with freshwater fish such as rainbow trout and channel catfish. Limited data are available on the effects of nitrite on seawater or euryhaline fish. The present study is the first attempt to outline in detail some hematological and metabolic changes in a seawater-adapted euryhaline fish, Lates calcarifer after prolonged and

acute nitrite exposure.

Based on the available data, the underlying toxicity mechanisms of nitrite cannot be fully worked out. However, the experimental results did not support the commonly accepted speculation that nitrite intoxicated fish died as a result of hypoxia induced by severe methemoglobinemia (Huey et al., 1980). In the first place, significant mortality was observed when Lates was exposed to comparatively low nitrite concentration at which methemoglobinemia was unlikely to occur. In the second place, hypoxic symptoms were not evident in Lates exposed to low nitrite level (10 mgl^{-1} NO_2-N) under which significant fish mortality was recorded. Remarkable hypoxic symptoms were only observed in fish subject to extremely high nitrite concentrations (up to 80 mgl^{-1} NO_2-N). It is speculated that other observed toxic effects such as gill failure and subsequent osmoregulation derangements probably play an important role in causing fish mortality.

In addition to the practical contribution to mariculture, academically, the present thesis also contributes to the general understanding of the mechanisms of nitrite toxicity in fish. Furthermore, the present study has laid a solid foundation and established guidelines for further investigations on this topic. Suggestions for further work are listed below:

(1) Elevation of serum sodium and potassium concentrations is

coupled with an inhibition in branchial Na^+-K^+-ATPase. Establishment of ultrastructural changes in gill tissue in response to nitrite exposure deserves further investigation in order to assess the impacts of nitrite on the osmoregulatory processes of fish.

(2) Increased production of ammonia was accompanied by a decrease in the activity of liver glutamate dehydrogenase. Assays of IMP level and AMP deaminase activity in both control and nitrite-treated fish are required to further confirm whether additional ammonia is produced by the purine nucleotide cycle.

(3) A higher blood oxygen affinity and a more hyperbolic blood oxygen dissociation curve in treated fish favour oxygen uptake from surrounding water across the respiratory surfaces. Higher blood oxygen affinity can arise from changes in number and percentage composition of hemoglobin fractions or erythrocyte ATP levels. Therefore, experiments on hemoglobin electrophoresis and erythrocyte organo-phosphate levels are worth studying.

(4) In general, the blood and tissue of nitrite-exposed freshwater fish accumulate nitrite so that the tissue nitrite concentration becomes higher than nitrite concentration in the water. However, this phenomenon is not observed in seawater fish. In seawater-adapted Lates,

the relatively small magnification of nitrite concentration in its blood stream probably contributes to its remarkable tolerance to nitrite toxicity as reflected by the high 96 hour LC50 value in sea water. However, the problem of whether freshwater-adapted Lates can still restrict the uptake of nitrite remains to be solved. In addition, the hematological and metabolic changes of Lates in response to nitrite exposure in fresh water deserve investigation.

(5) Methemoglobin formation resulted in a reduction in blood oxygen carrying capacity. The effect, if any, of decreased blood oxygen capacity on the oxygen utilization of various organs remains to be studied.

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